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Porcine pancreatic α -amylase hydrolysis of modified substrates and the specificity of subsite binding

Paul J. Braun
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PORCINE PANCREATIC ALPHA-AMYLASE HYDROLYSIS OF MODIFIED
SUBSTRATES AND THE SPECIFICITY OF SUBSITE BINDING

Iowa State University

PH.D. 1984

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Porcine pancreatic α -amylase hydrolysis
of modified substrates and the specificity
of subsite binding

by

Paul J. Braun

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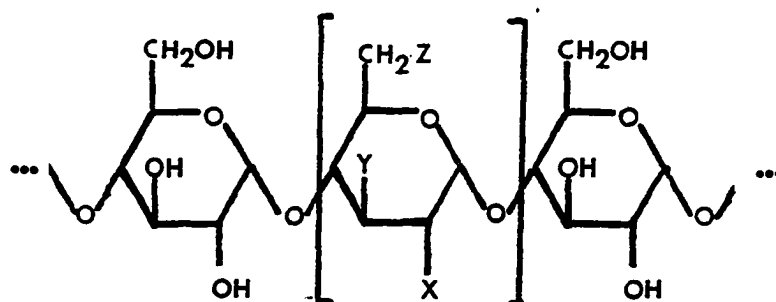
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ABBREVIATIONS USED

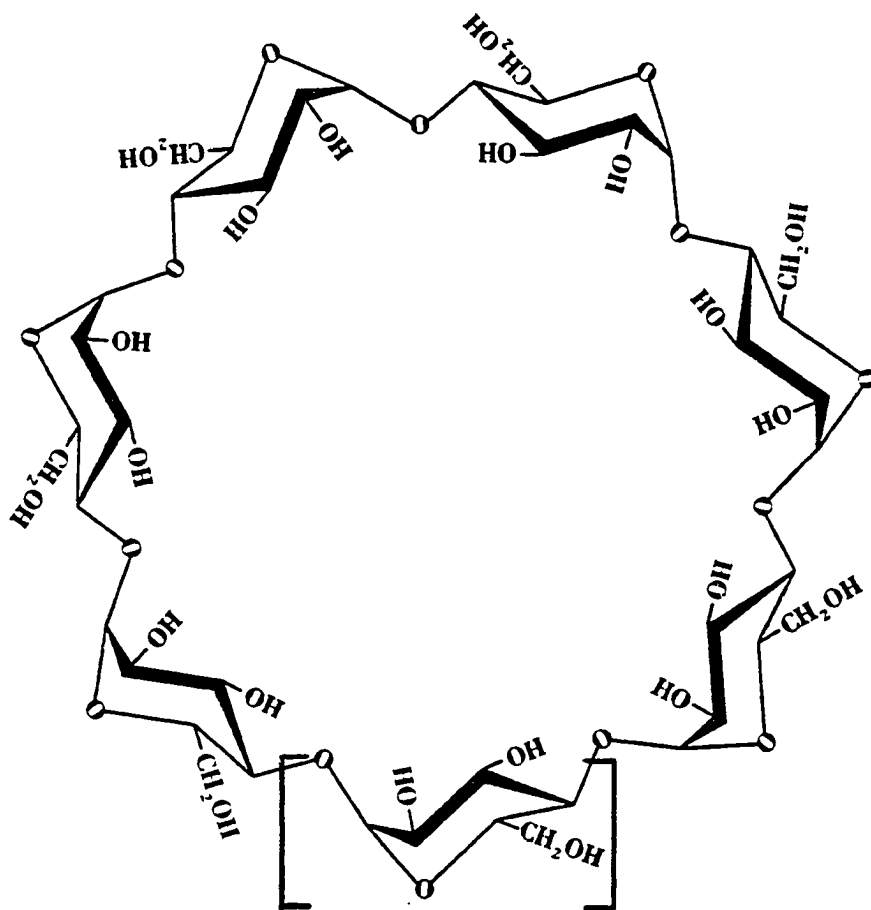
Å	Ångstrom(s)
CCl ₃ F	fluorotrichloromethane
cm	centimeter(s)
DEAE-	diethylaminoethyl-
2d-G ₃	2-deoxymaltotriose
2dGlc	2-deoxy-D-glucose
D ₂ O	deuterium oxide
d.s.	degree of substitution
¹⁹ F-n.m.r	fluorine-19 nuclear magnetic resonance
G ₁	D-glucose
h	hour(s)
[³ H]-	tritium
Hz	hertz
J	coupling constant
L	liter(s)
l.c.	high-pressure liquid chromatography
M	molar
mCi	millicurie(s)
mg	milligram(s)
MHz	megahertz
min	minute(s)
mL	milliliter(s)
mM	millimolar

mmole	millimole(s)
m.p.	melting point
PAC	polar amino cyano
PPA	porcine pancreatic α -amylase
ppm	parts per million
R_G	chromatographic mobility relative to D-glucose
sec	second(s)
TFA	trifluoroacetic acid
tris-HCl	tris(hydroxymethyl)aminomethane- hydrochloride
t.l.c.	thin-layer chromatography
U	International Unit(s) of enzyme activity
UDP-	uridine diphospho-
UDPG	uridine diphosphoglucose
μ L	microliter(s)
μ M	micromolar
ϕ_c	chemical shift (fluorine-19)

STRUCTURES OF MODIFIED SUBSTRATES



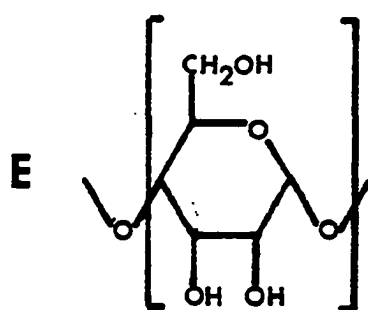
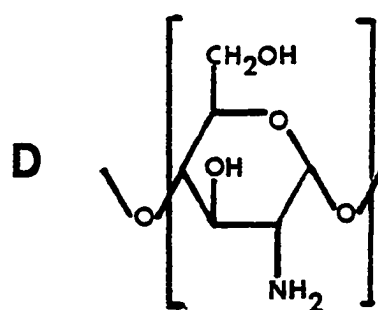
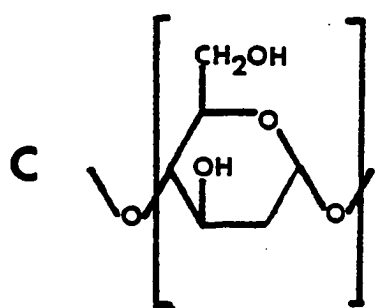
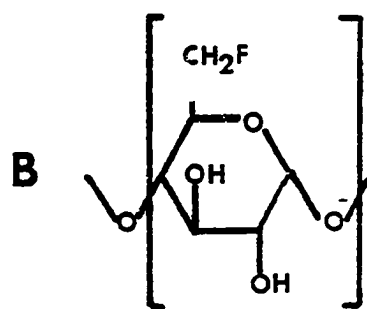
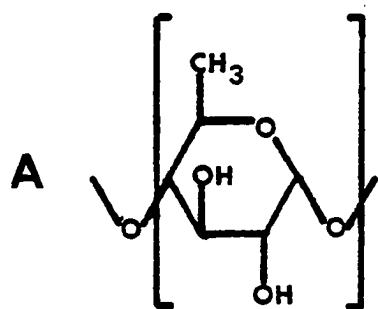
General structure of modified amyloses. These substrates typically had a degree of substitution (d.s.) of 0.15 or less, indicating that 15% or less of the glucose residues were modified. Modifications at C-2, C-3, and C-6 result from alterations at X, Y, and Z, respectively.



General structure of modified β -cyclodextrins. These substrates contained modifications at a single D-glucose residue, as indicated by brackets.

Structure of modified D-glucose residues. A) 6-deoxy-D-glucose;
B) 6-deoxy-6-fluoro-D-glucose; C) 2-deoxy-D-glucose; D) 2-amino-2-
deoxy-D-glucose; E) D-allose.

x



GENERAL INTRODUCTION

Porcine pancreatic α -amylase (PPA) is a member of a large class of enzymes (the amylases), which catalyze the hydrolysis of starch, amylose, amylopectin, and other α -(1 \rightarrow 4)-linked glucans. The principal unbranched products formed from extensive hydrolysis with PPA are maltose and D-glucose. The enzyme active site (Fig. 1) has been shown to contain five subsites, which bind a series of five consecutive D-glucose residues, with cleavage occurring between the second and third residues from the reducing end (1).

Although this enzyme has been widely studied, a clear picture of its role in catalysis has not yet emerged. It is believed that enzymes, like other chemical catalysts, lower the activation energy necessary to transform the substrate into a crucial high-energy species en route to product formation. The high energy species corresponding to this energy maximum is called the transition state. Any reduction in the activation energy will produce an accompanying increase in the rate of reaction.

The transition state for reactions catalyzed by amylases and other carbohydrases is believed to be similar to that proposed for acid-catalyzed hydrolysis of glycosides (Fig. 2). This transition state is usually depicted as an oxycarbonium ion in a planar half-chair or sofa conformation (2-6). Mechanisms by which carbohydrases might assist in forming or stabilizing this transition state have not been clearly identified. Some factors which have been suggested to account for this assistance are acid-base catalysis (7), electrostatic stabilization (8), tor-

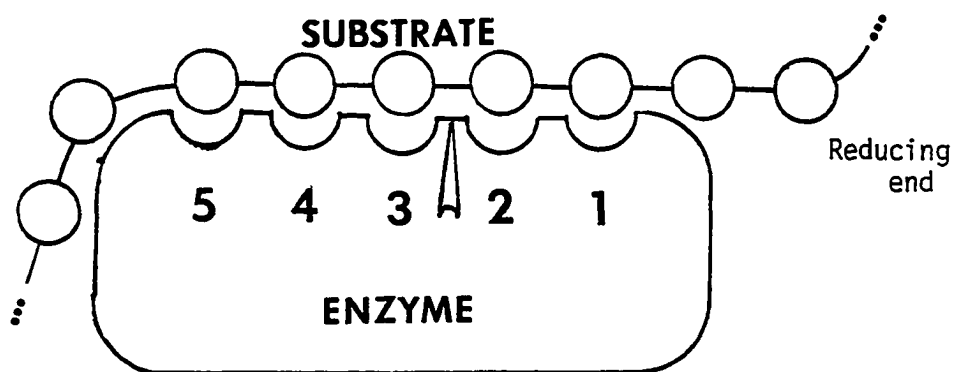
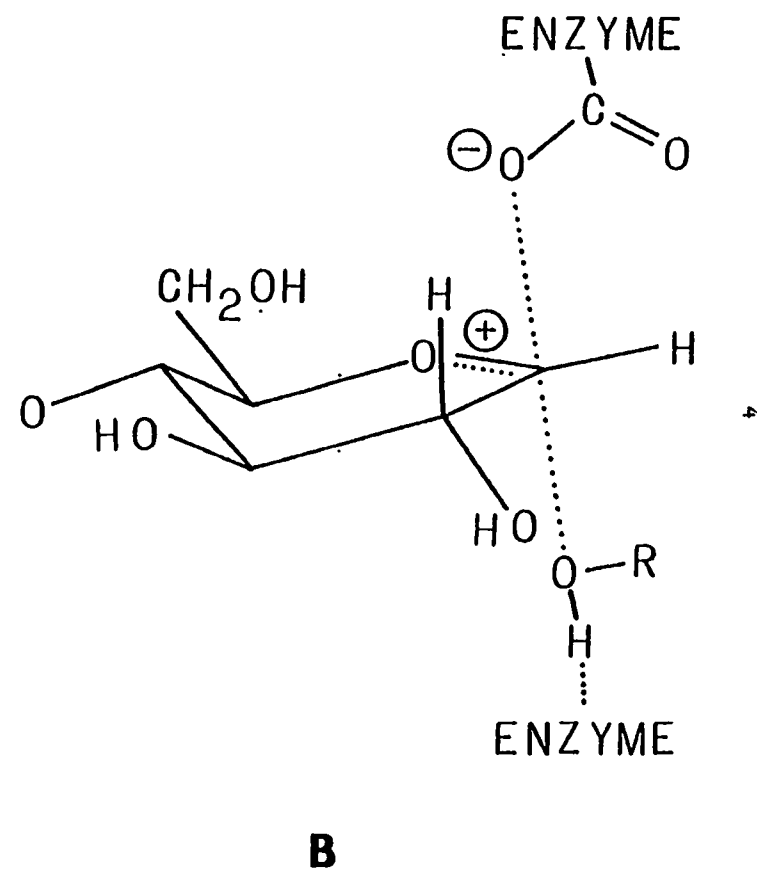
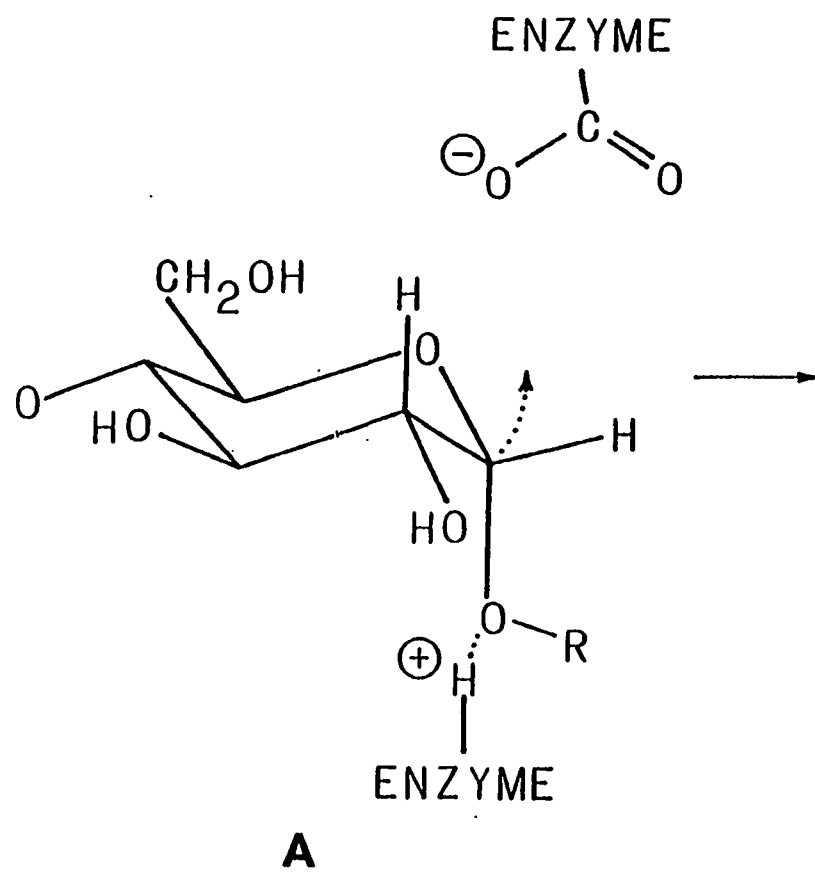


Figure 1. Pictorial representation of porcine pancreatic α -amylase active site. Circles represent D-glucose residues. Numbers refer to active site subsites, with the point of hydrolysis indicated by an arrow.

Figure 2. Proposed formation of half-chair transition state by porcine pancreatic α -amylase. A) Binding of substrate in full-chair ground state, showing protonation of the substrate glycosidic linkage by a basic group on the enzyme. B) Formation of the half-chair oxycarbonium ion. This transition state may be directly stabilized during the course of the reaction by the presence of a negatively-charged enzyme functional group, or may collapse to produce an enzyme-substrate covalent intermediate.



sional strain (9), and binding site complementarity (10). Different roles have been proposed for this transition state with respect to the overall enzyme reaction mechanism, as well. One mechanism proposed for amylases has described this transition state as the energy maximum for an S_N2 -like inversion leading to formation of a covalent enzyme-substrate intermediate (4-5). For α -amylases, this would be followed by a second inversion reaction leading to product formation with retention of configuration. A second mechanism, similar to that proposed for lysozyme, depicts the transition state as being directly stabilized by electrostatic forces without the formation of covalent intermediates; the glycosidic bond is cleaved, while the leaving group diffuses out of the active site and water diffuses in to give hydrolysis (2-3).

One method which may be used to identify important interactions between enzyme and substrate involves the use of modified substrates. The action of PPA on modified substrates gives products with structures which reflect any serious disruption of enzyme-substrate interactions. In other words, modification of D-glucose residues may result in lack of interaction or unfavorable interaction with a particular enzyme subsite, so that productive binding (binding leading to hydrolysis) does not occur. The absence of productive binding at that subsite will be reflected in the structure of the resulting oligosaccharide products. Therefore, analysis of the structure of modified oligosaccharides obtained from the action of PPA on modified substrates allows identification of subsites which are sensitive to that modification. By using substrates in which only a small percentage of the D-glucose residues contain a given modification, it is

possible to isolate the effect of a single substituted group on enzyme-substrate binding.

The action of PPA on several modified substrates has been examined (11-21). Usually, these have been substrates containing bulky substituents. The amylose analogs described in this work all contain modifications which are approximately the same size as the hydroxyl group, or smaller. They fall into three categories: substrates with small modifications (amylose analogs containing 2-deoxy-, 6-deoxy-, and 6-deoxy-6-fluoro-D-glucose), substrates with altered stereochemistry (amylose containing D-allose), and substrates with charged groups (glycogen containing 2-amino-2-deoxy-D-glucose). In each instance, the degree of substitution (d.s.) was approximately 0.15 or less, indicating that no more than 15% of the total number of D-glucose residues were modified.

In some of these experiments, modified cyclodextrins have been used as substrates. Cyclodextrins (cyclomaltodextrins, cycloamyloses, Schar-dinger dextrins) are a family of cyclic α -(1 \rightarrow 4)-glucans produced by bacterial enzymes (22). The most abundant of these are α -cyclodextrin and β -cyclodextrin, which contain six and seven D-glucose residues, respectively. β -Cyclodextrin is a poor substrate for PPA and also acts as an inhibitor (23). There are some advantages, however, to using these compounds. Using chemical synthesis, it is possible to produce pure modified cyclodextrins with a single defined structures. Modified amyloses, on the other hand, can be characterized only as average structures, and the products of undesired side reactions occurring during chemical synthesis cannot always be removed from the polymer. The cyclodextrins are soluble

in a wider variety of organic solvents than amylose, and some control of reaction regioselectivity and stoichiometry are possible under appropriate conditions (24-26). Modified cyclodextrins may be prepared through enzymatic reactions as well (27).

The use of modified substrates should provide information about several aspects of α -amylase binding requirements. 1) By comparing the results obtained from bulky substituents to results from small substituents, it should be possible to locate sites which are sensitive to steric disruption. 2) By replacing substrate hydroxyl groups with hydrogen or fluorine, it should be possible to determine whether hydrogen bonding is an important driving force in binding and/or catalysis. 3) By observing the effects of inversion of stereochemistry, the role of hydroxyl group configuration may be determined. Observing the effect of D-allose residues on substrate hydrolysis may also supply information about steric interactions which are affected by introduction of an axial hydroxyl group. 4) By using substrates containing charged modifications, it should be possible to determine whether PPA action may be seriously disrupted by changes in electronic interactions.

There are several reasons for performing these studies using amylases. These enzymes are of considerable economic importance; the enzymes used in starch conversion (amylase, amyloglucosidase, and glucose isomerase) account for 40% of the total production of enzymes for industrial use (28). They are widely used in the brewing and corn-sweetener industries, and have been used experimentally in biomass conversion schemes. Human pancreatic and salivary α -amylases are clinically impor-

tant as diagnostic markers for pancreas and parotid function (29-30). The potential for commercial and medical exploitation arising from basic research on amylases is illustrated by the brief success of α -amylase inhibitors (so-called "starch blockers") as diet aids. Knowledge about the action of amylases may also extend, to some degree, to carbohydrate-processing enzymes in general, including lysozyme, cellulases, and others.

Porcine pancreatic α -amylase is well-suited to these studies for other reasons. It is readily commercially available, and has been widely studied, so that there exists a large body of literature regarding the enzyme action and properties. In addition, the five-subsite active site is small enough so that the effects of substrate modification may be conveniently mapped over most or all of the enzyme active site. Active sites for other carbohydrases are as large as 9-10 subsites, as seen for *Bacillus subtilis* α -amylase (31-32).

The goal of the experiments described in this dissertation has been to identify basic chemical forces driving α -amylase catalysis. Since these studies have required the use of a variety of carbohydrate hydrolases and synthases, the results may also point to common features of carbohydrase mechanism, which could be applicable to a broad class of enzymes.

Explanation of Dissertation Format

The experimental work in this dissertation is presented in three sections. Each of these sections represents an individual paper which has been or will be submitted to a scientific journal for publication. The work in all three sections was performed entirely by myself, under the guidance of Dr. Dexter French and Dr. John Robyt. These papers were prepared by myself, with assistance from Dr. Robyt in editing and revising the manuscripts.

LITERATURE REVIEW

Properties, Action Pattern, and Mechanism of Porcine Pancreatic α -Amylase

The properties and mechanisms of the amylases, including PPA have been reviewed extensively (2-5,33-37). Isolation of crystalline PPA was first described by Meyer et al. in 1946 (38-39), and improved preparative methods were later published by Fischer and Bernfeld (40), Caldwell et al., (41), and Loyter and Schramm (42). The enzyme was first reported to be a homogeneous enzyme by free boundary and paper electrophoresis (38,39,41), with an estimated molecular weight of 45,000 daltons as determined by sedimentation and diffusion methods (43). Subsequent isolation by DEAE-cellulose chromatography and disc gel electrophoresis revealed the presence of two isoenzymes, with different isoelectric points (44-47). A partial amino acid sequence, obtained from trypsin proteolysis fragments, was determined for isozyme II (47-49), and the complete sequence for isozyme I has been deduced from proteolytic and cyanogen bromide fragments (50-52). The isozymes are similar or identical with respect to their amino acid composition and molecular weight (51,500 daltons, 460-496 amino acids), with the same C-terminal amino acid and N-terminal sequences (46-52). It has been suggested that the isozymes may actually be artifacts, caused by deamination of a single molecular species during separation (47,53). The enzyme sequence shows a high degree of homology with mouse salivary and liver α -amylases (54), and small regions of the enzyme may be conserved for several bacterial and mammalian amylases (55).

Porcine pancreatic α -amylase is a single polypeptide chain containing two thiol groups and either four or five disulfide bridges (47,56). The enzyme has been shown to possess two binding sites for the poor substrate, maltotriose (57), and at least two binding sites for β -cyclodextrin (58). Further evidence for the existence of two or more binding sites is provided by the formation of multimolecular amylase-glycogen complexes (42). Low angle x-ray scattering, x-ray diffraction, and hydrodynamic studies suggest that the molecule consists of two structural domains separated by a large crevice (58-61). It has been proposed that the enzyme is organized into two similar or identical domains formed by a tandemly repeated sequence. This suggestion was based on the observation of dihedral symmetry detected in early x-ray diffraction studies (61), and on experiments in which proteolysis of the enzyme, followed by reduction with dithiothreitol yielded two catalytically active fragments with molecular weight of approximately 25,000 daltons, which have identical peptide maps (62-63). X-ray studies (5 Å resolution) have indicated that the two domains are of unequal size (59), and the existence of a tandemly repeated peptide chain is not supported by the published amino acid sequence (49,52).

Like other α -amylases, PPA is a calcium metalloenzyme having one gram-atom of firmly bound calcium per mole of enzyme, and as many as 9-10 loosely bound calcium ions (64). The firmly bound calcium apparently contributes to maintaining the proper tertiary structure, and stabilizes the enzyme against denaturation and proteolysis (65,66). Chloride ion (10 mM) is also required as an allosteric activator for maximum enzyme activ-

ity (38,67), although bromide and other anions may give partial activation (68). The optimum pH range lies between 6.5 and 6.9 (38), and the enzyme loses activity rapidly above 50° (33).

The action of PPA may be classified by some general characteristics. Enzyme hydrolysis of polymeric substrates produces a rapid drop in viscosity, resulting from endo-attack (hydrolysis of interior glycosidic linkages) of polymeric chains. Amylases of this type are known as liquifying amylases, as opposed to saccharifying amylases, which proceed via endwise (exo-) attack of glucan chains. PPA also produces products with the α -anomeric configuration, hence the designation α -amylase.

More detailed information regarding the action pattern of PPA has been obtained from a number of studies. Early experiments (69) observing differences in plots of reducing value versus iodine complex blue value for various α -amylases were explained in terms of different size distributions of reaction products. It was later shown, however, that when γ -cyclodextrin is used as a substrate for PPA in the presence of excess β -amylase, maltotriose is formed in the very early stages of reaction (70). This was interpreted as evidence for a repetitive (or multiple) attack mechanism, in which the enzyme may remain complexed with substrate and cleave it several times in a single encounter. A later study by Robyt and French (71), which compared hydrolysis by several amylases with acid hydrolysis, estimated the degree of multiple attack of PPA to be 6. An alternative explanation for these results was forwarded by Banks et al. (72), which suggested that the enzyme was composed of two isozymes or had two distinct hydrolytic activities. They later agreed (73) that PPA does

seem to operate through a multiple-attack mechanism. This mechanism was further supported by later studies by Thoma (74) and Hutny and Ugorski (75). The mathematical models employed in several of these studies have recently been reexamined and slightly refined (76,77). A theoretical approach for distinguishing single and dual active site models for PPA which relates these models to the multiple-attack mechanism has also been described (78).

Studies using maltooligosaccharides radiolabeled at the reducing-end and at the nonreducing-end glucose residues demonstrated that after an initial bond cleavage, multiple attack proceeds toward the nonreducing end (79). Amylolysis of reducing-end labeled oligosaccharides also provided evidence for an active site model containing five glucose-binding subsites (1). It was observed that maltopentaose was initially cleaved by the enzyme exclusively between the second and third residues from the reducing end. Higher oligosaccharides had more than one cleavage point, but cleavage was highly favored at positions which allowed binding of five subsites in the same manner as maltopentaose. Glucose was produced only from hydrolysis of maltotetraose or maltotriose. It has also been observed that the reaction velocity increases with increasing substrate size for substrates containing three to five glucose residues, but that reaction velocity remains nearly constant for maltopentaose, maltohexaose and maltoheptaose (80). On the basis of these results, an active site model was proposed by Robyt and French which consists of five subsites, with cleavage occurring between the second and third subsites from the reducing end, as depicted in Fig. 1. A method for calculating binding

energies for individual subsites of depolymerases has been developed (32,81-84), but this method is not readily applicable if multiple attack is occurring (81). Subsite binding energies have been calculated for several bacterial and fungal amylases (32,81,82,84,85). Subsite binding energies have also been reported for PPA (80,86). One of these reports, however includes no experimental detail (85), and the other (80) contains serious experimental and theoretical flaws (J. A. Thoma, Department of Chemistry, University of Arkansas, personal communication). Partial calculations for PPA have also been performed in our laboratory (21).

The mechanism of amylase-catalyzed hydrolysis has been proposed to resemble acid-catalyzed hydrolysis of glycosides (2-5). The transition state for the acid-catalyzed reaction mechanism is usually represented as a half-chair oxycarbonium ion, which is stabilized by partial negative charges on leaving and attacking groups (6,87). Support for this type of mechanism among carbohydrases has been provided by the observed inhibition of PPA and related enzymes by gluconolactone and maltobionolactone (88); and by kinetic isotope effects (89), dynamic n.m.r. ring-flattening studies (90), and results of x-ray diffraction studies for lysozyme (9). Recent binding studies and conformational energy calculations for lysozyme have suggested, however, that oligosaccharides may be bound to the enzyme without appreciable strain (91-95).

At least two basic types of mechanisms have been proposed for the α -amylases, which may be distinguished by the presence or absence of covalent enzyme-substrate intermediates (2-5). Both mechanisms suggest that assistance in formation or stabilization of a half-chair transition state

is important to amylase catalysis. A number of factors have been proposed to account for this assistance. Acid-base catalysis by amino acid side chains is one of the most widely-cited causes of rate acceleration (2-5,7). Present evidence seems to implicate carboxylate and imidazolium groups as the most probable catalytic groups at the active site of PPA. These assignments were originally made on the basis of pH data (67,96), absence of phosphate in the enzyme (44), lack of inactivation in presence of thiol-modifying reagents (97), and photooxidation data (67). A later series of papers by Hoschke et al. (98-99) and Holló et al. (100) using modifying reagents for amine, tyrosine, histidine, tryptophan, and carboxyl groups has supported these assignments, and has also identified groups which are important to subsite binding throughout the PPA active site. The catalytic groups have commonly been shown as participating in the formation of enzyme-substrate intermediates (4-5), although such intermediates have not been isolated. An alternative mechanism has been proposed (2-3), in which charged catalytic groups stabilize an oxycarbonium ion through electrostatic interactions without formation of a covalent intermediate (8). Enzyme-induced distortion of substrate has also been suggested as a driving force for catalysis. This proposal has been based mainly on the results of x-ray diffraction of lysozyme (9). Other effects also have been proposed as contributing factors, including entropy effects (2) and solvation effects (101).

PPA Hydrolysis of Substrates Other Than Amylose

Because of the limited chemical techniques available for modifying polysaccharides and the tremendous difficulties involved in chemical synthesis of oligosaccharides from monomers, many studies of PPA action have relied on use of naturally occurring polysaccharides. Such studies have examined polysaccharides containing 6-O-methyl- groups (12), α -(1 \rightarrow 3)- (17) and α -(1 \rightarrow 6)-branched linkages (13,14,18), and 3-O- and 6-O-phosphate esters (19). Other modified amylase substrates have been prepared by enzymatic methods, chiefly through the use of *Bacillus macerans* glucanotransferase. This approach has been used to obtain coupling products containing sucrose, 3-O-methyl-maltose, methyl-6-deoxy- β -glucopyranoside, and methyl- and phenyl-D-glucopyranosides, as well as other products (21,102,103).

Studies on amylase substrates modified by chemical techniques largely have been confined to amyloses modified at C-6. Weill and coworkers have examined a number of amylase substrates modified at C-6, including 6-O-methylamylose (11), 6-deoxy-6-iodoamylose (104), 6-amino-6-deoxyamylose (15), and 6-deoxyamylose (105). These studies have focussed mainly on the action of amylases from *Bacillus subtilis*. Bines and Whelan (106) published a preliminary report of the hydrolysis of 6-deoxyamylose and 3,6-anhydroamylose by human salivary α -amylase. This report was unusual, in that it tentatively identified a modified monosaccharide (6-deoxy-D-glucose) as a product of amylase hydrolysis. A few experiments have also been performed on substrates containing chemical modifications at second-

ary hydroxyls. The most comprehensive of the studies on PPA is probably the study by Chan et al. (20), which examined the effect of hydroxyethyl groups at C-2, C-3, and C-6 of the amylose molecule. Kainuma and French have also studied amylose which was first oxidized by periodate and then reduced with sodium borohydride as a PPA substrate (16). This type of chemical modification results in a derivative which lacks a bond between C-2 and C-3.

Experiments on the action of PPA reveal consistent trends related to the effect of sterically large substituents (12,14,17,18,20). Subsite 3 is very sensitive to bulky modifications at C-2, C-3, and C-6; subsite 2 is sensitive to bulky modifications at C-2; and subsite 4 is sensitive to bulky modifications at C-6. Subsites 1 and 5 seem to be less susceptible to effects of bulky modification, although subsite 5 has not been as rigorously studied as the other four subsites (20). Presence of a charged phosphate at C-6 gives results similar to those obtained for bulky substituents, but the effect of a phosphate group on C-3 seems to be much more disruptive than that of a bulky, uncharged large substituent (19). Oxidized-reduced glucose residues may be productively bound at subsites 1 and 5, but not at subsites 2, 3, and 4 (16).

Synthesis of Selected Modified Amyloses and Amylose Analogs

The only reported chemical synthesis of deoxy- α -(1 \rightarrow 4)-glucan has been the synthesis of 6-deoxyamylose described by Bines and Whelan (106) and Weill et al. (105), via sequences involving tosylation, iodination, and

reduction with either lithium aluminum hydride or sodium borohydride. A series of papers by Zemek, Kučár, and coworkers have described the incorporation of 2-deoxy-, 3-deoxy-, 4-deoxy-, and 6-deoxy-D-glucose into glycogen by incubating deoxysugar-1-phosphates with potato phosphorylase (107), or by incubating uridine diphosphate (UDP) nucleotides of deoxy-sugars with UDP-glucose-glycogen glycosyl transferase (108-110). For both systems, the authors found that the rate of hexose incorporation decreased in the following order: D-glucose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, 4-deoxy-D-glucose, and 3-deoxy-D-glucose. They also observed that consecutive residues of 2-deoxy- and 6-deoxy-D-glucose could be added to the nonreducing termini of the glycogen chains.

No practical chemical approaches to synthesis of fluorinated polysaccharides have been reported. Reaction of starch with hydrofluoric acid at 30°, or reaction of 6-O-tosylated starch with potassium fluoride in refluxing ethane-1,4-diol, have been reported to give starch containing 6-deoxy-6-fluoro-D-glucose, 6-deoxy-D-xylo-hexos-5-ulose, and other products (111). Analysis of these products proved difficult since they were not readily hydrolyzed by acid, even under drastic conditions. An extensive review of current approaches to fluorination of saccharides has been published (112). Most of these approaches, however, are designed for monosaccharide synthesis, and are not readily adapted for polysaccharide modification.

The introduction of fluorosugars into polysaccharides by enzymatic methods has not been studied extensively. The formation of UDP-2-deoxy-2-fluoro-D-glucose and UDP-2-deoxy-D-mannose, and the incorporation of

these sugars into glycoproteins has been reported in yeast and chick embryo cells (113). Other than this report, and another which examined various fluorosugars as substrates for hexokinase (114), little work has been completed regarding the metabolism of fluorosugars by enzymes involved in synthesis of α -(1 \rightarrow 4)-glucans.

The chemical synthesis of a modified amylose with inverted stereochemistry at C-3 has been described by Kondo and Takeo (24). Amylose (or β -cyclodextrin) containing D-allose residues was produced by selective benzylation leaving C-3 unprotected. The C-3 hydroxyl group was then oxidized to form the 3-ketone, which was reduced to give D-allose. This product also contained a very small amount of D-mannose. Although a procedure has been reported for obtaining amylose selectively oxidized at C-2 (115,116), reduction using conventional reagents such as sodium borohydride favors overall retention of configuration, rather than inversion (117).

Studies synthesizing or using amylose products of these types have been limited. Chemical synthesis of modified amyloses is severely restricted by a number of factors, some of which are readily apparent. Amyloses are highly insoluble in most organic solvents, and contain three chemically similar hydroxyl groups that are often difficult to selectively modify, especially at the secondary hydroxyls. Ideally, the modified amylose should contain a minimum of side products, which requires that only high-yielding, specific reactions may be used. Analysis of reaction scheme intermediates and final products also poses special problems for these modified amyloses. Other limitations are less obvious. Reaction

sequences involving S_N2 displacements at C-2 or C-3 are generally unsuitable, since S_N2 reactions are highly unfavorable at C-2 for nearly all sugars, and are unfavorable at C-3 for α -glucosides (118). Polymer cross-linking may also be a serious problem for S_N2 reactions involving efficient leaving groups, which may result in formation of extremely insoluble products. Enzymatic synthesis of modified products may provide a useful alternative to chemical methods, but these techniques depend entirely on the existence of enzyme(s) having the appropriate substrate specificity required for the desired modification.

SECTION I.

PORCINE PANCREATIC α -AMYLASE HYDROLYSIS OF SUBSTRATES CONTAINING
6-DEOXY-D-GLUCOSE AND 6-DEOXY-6-FLUORO-D-GLUCOSE
AND THE SPECIFICITY OF SUBSITE BINDING

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ABSTRACT

Hydrolysis of 6-deoxyamylose and mono-6-deoxy-6-fluoro- β -cyclodextrin by porcine pancreatic α -amylase (PPA) produces low-molecular-weight modified products, which have been analyzed by chemical and chromatographic techniques. Results for both substrates show that modified D-glucose and two isomers of modified maltoses are produced in the enzyme reaction. In addition, the formation of maltoses which are modified in the nonreducing residue is more favored than the formation of maltoses modified in the reducing residue. These results indicate that productive binding of 6-fluoro- and 6-deoxy-D-glucose residues is permitted at subsites 1 through 4 of the PPA active site but that binding of these modified residues may be less favorable at subsite 3, the subsite at which catalytic attack occurs.

INTRODUCTION

The action of porcine pancreatic α -amylase (PPA) on branched and modified substrates has been observed in a number of studies (1-8). In nearly all instances, these substrates have contained either bulky or charged substituents. Hydrolysis products from these substrates have included modified disaccharides, trisaccharides, and larger products. Bines and Whelan have reported (9), in a preliminary study on human salivary amylase, that a product with the paper chromatographic mobility of 6-deoxy-D-glucose is obtained from the enzymolysis of 6-deoxyamylose. Because previous studies on PPA hydrolysis of modified substrates have not observed the formation of modified monomers, we have examined the effects of small substituents on PPA action more closely. Inasmuch as fluorine is smaller than the hydroxyl group, is similar to oxygen in electronegativity, and has non-bonded electrons that might participate in hydrogen bonding (10), mono-6-deoxy-6-fluoro- β -cyclodextrin was used as one substrate for the enzyme. Like native β -cyclodextrin, the fluorinated derivative is a relatively poor substrate for PPA. Fluorinated β -cyclodextrin may be readily synthesized and purified, whereas synthetic difficulties and the formation of side products make the use of fluorinated amylose difficult. The hydrolysis of 6-deoxyamylose by PPA also has been examined. Our results may be interpreted in terms of the ability of PPA to productively bind modified sugars at the five subsites of the enzyme active site. Using this approach, it may be possible to assess the importance of the C-6 hydroxyl group for enzyme-substrate interactions. These

results may also be compared with those obtained for substrates containing bulky modifying groups (e.g., hydroxyethyl, α -D-glucosyl, and phosphate) at C-6, in order to identify sites where enzyme-substrate interactions are sensitive to steric disruption.

EXPERIMENTAL

Materials

Amylose (Superlose from Stein-Hall Co.) and PPA (Boehringer-Mannheim, 10,000 U/ml) were commercial samples. The PPA contained no detectable α -glucosidase activity as assayed by using *p*-nitrophenyl- α -D-glucoside. β -Cyclodextrin was prepared by the method of French et al. (11).

Methods

Ascending and descending paper chromatography were performed on Whatman 3MM paper by using 1-propanol:water, 7:3 (solvent A) or nitromethane:ethanol:acetic acid:saturated boric acid, 8:1:1:1 (12) (solvent B). Reducing sugars were detected by the silver nitrate dip method (13). Total carbohydrate was determined by using the orcinol-sulfuric acid method, as adapted for use with a Technicon AutoAnalyzer (14). Enzymolysis products were reduced with sodium borohydride by the procedure of Sloneker (15). Acid hydrolysis was performed in 3 M trifluoroacetic acid (TFA) at 100° for 2 h in sealed ampules, unless otherwise indicated. After hydrolysis, samples were cooled, and the acid was removed by repeated evaporation under reduced pressure. Reducing sugars and sugar alcohols were identified by paper or liquid chromatography using authentic materials as standards. Tritiated samples on paper chromatograms were detected by autoradiography after spraying with En³Hance (DuPont-NEN).

Charcoal chromatography columns were prepared by the method of Chan et al. (8). High-pressure liquid chromatography (l.c.) employed a Waters liquid chromatograph with refractive index detector. Analytical separations were performed on Whatman PXS 10/25 polar amino cyano (PAC) columns, with 85% acetonitrile at a flow rate of 1.0 mL/min as solvent. Fluorinated β -cyclodextrin was purified on a Whatman M-9 PAC semi-preparative column operated at 3.0 mL/min with acetonitrile:water:tetrahydrofuran, 70:27:3, as solvent. ^{19}F -n.m.r. spectra were obtained on a Bruker 300 MHz instrument.

Substrates

Samples of 6-deoxyamylose with degrees of substitution (d.s.) of 0.15 and 0.50 were prepared by the method of Weill et al. (16), via 2,3-di-O-acetyl-6-O-toluenesulfonylamylose.

Monofluorinated β -cyclodextrin was prepared via the corresponding mono-toluenesulfonyl (tosyl) ester. Mono-6-O-tosyl- β -cyclodextrin was prepared from β -cyclodextrin (35 g) by a modification of the procedure of Omichi et al. (17). After treatment with tosyl chloride, the reaction mixture was concentrated to a thick syrup and poured into 2-propanol. The precipitate was filtered and recrystallized from warm water to give a crude product (20.9 g). A portion of this crystalline material (4.0 g) was dissolved in warm water (3 L) and chromatographed at 40° on a charcoal column (4.8 x 30 cm) by eluting successively with water (4 L), 10% ethanol (2 L), 15% ethanol (2 L), 20% ethanol (4 L), 30% ethanol (4 L), and 25% 1-

propanol (3 L). The 30% ethanol fraction was evaporated to give pure mono-6-O-tosyl- β -cyclodextrin (2.3 g). Fluorinated β -cyclodextrin was prepared by refluxing mono-6-O-tosyl- β -cyclodextrin (4.4 g) with anhydrous potassium fluoride (1.5 g) in ethane-1,2-diol (135 mL) for 3 min. The cooled reaction mixture was poured into 2-propanol, and the precipitate was removed by centrifugation. Repeated recrystallization from hot water gave mono-6-deoxy-6-fluoro- β -cyclodextrin (600 mg) containing small amounts of an impurity. Further purification by l.c. yielded pure product, m.p. >200°, with an ^{19}F -n.m.r. spectrum similar to that of 6-deoxy-6-fluoro-D-glucose (18) (D_2O , CClF_3 external standard, $\phi_{\text{C}} = 234.3$ ppm, J_{FH} (geminal) = 47.9 Hz, J_{FH} (vicinal) = 29.3 Hz). Acid hydrolysis of this material followed by paper chromatography (solvent A, 1 ascent) identified 6-deoxy-6-fluoro-D-glucose, D-glucose, and oligosaccharide products. Elemental analysis: calc. for $\text{C}_{42}\text{H}_{69}\text{O}_{34}\text{F}$: C, 44.37; H, 6.07; F, 1.67. Found: C, 44.28; H, 6.37; F, 1.76.

Enzyme Hydrolysis

6-Deoxyamylose (d.s.=0.15, 1.0 g) was dissolved in dimethyl sulfide/water, 9:1 (15 mL), and buffer (15 mL) containing 200 mM sodium glycerophosphate, 100 mM sodium chloride, and 0.2% sodium azide was added. The pH of the solution was adjusted to 6.9, and it was diluted with water to a final volume of 150 mL. This solution was filtered and warmed to 37°, and a solution of PPA (15 mL, 650 U/mL) containing 20 mM sodium glycerophosphate and 10 mM calcium chloride, pH 6.9, was added. After 3

days, a sample was removed and analyzed by paper chromatography (solvent A, 2 ascents), which showed D-glucose and maltose as the only major products. The enzyme digest was then applied to a charcoal column (4.8 x 30 cm) and eluted with water (3 L), then with a gradient of 0-8% t-butyl alcohol. Fractions (25 mL) were collected, and every other fraction was analyzed for total carbohydrate. Every tenth fraction was also analyzed by paper chromatography (solvent A, 2 ascents).

Mono-6-deoxy-6-fluoro- β -cyclodextrin (100 mg) was dissolved in 9.0 mL of warm water, and 1.0 mL of buffer (200 mM sodium glycerophosphate, 100 mM sodium chloride, 0.2% sodium azide, pH 6.9,) was added. An enzyme solution was prepared by centrifuging 1.0 mL of suspended PPA. The pellet was dissolved in 3.0 mL of 20 mM sodium glycerophosphate containing 10 mM calcium chloride at pH 6.9. This enzyme solution (3.0 mL) was added to the substrate solution (10.0 mL) and was allowed to react at 37° for 5 days. Samples (50 μ L) were removed daily and examined by paper chromatography (solvent A, 2 ascents). An additional sample (400 μ L) was removed at the end of the reaction time, and the remaining solution was applied to a charcoal column (2.4 x 30 cm). This column was eluted with water (1 L), and 1%, 2%, 3%, 4%, and 7% t-butyl alcohol (500 mL each). Each eluate was collected as a single fraction, evaporated to dryness, and dissolved in 400 μ L of water.

RESULTS

PPA Hydrolysis of 6-Deoxyamylose

Two major products were obtained from charcoal chromatography. These were identified as D-glucose, which eluted in the water fraction, and maltose, which eluted near the beginning of the t-butyl alcohol gradient. Several minor products also were detected by paper chromatography. Three of these products (designated 6-DG, 6-DM, and 6,6-DM) were tentatively identified as low-molecular-weight 6-deoxy-oligosaccharides, based on their paper chromatographic mobilities and longer retention on charcoal columns. The first of these compounds, 6-DG, had a paper chromatographic mobility relative to D-glucose (R_G) of 1.23, identical to that of 6-deoxy-D-glucose. This compound eluted from charcoal at the beginning of the t-butyl alcohol gradient along with maltose and was detected only in small amounts. The second of these components had an R_G value similar to D-glucose ($R_G = 0.99$), but eluted from the charcoal column after maltose. The paper chromatographic mobility of this compound was consistent with that predicted for mono-6-deoxymaltose (19). Relatively larger amounts of this product were formed. The third product, 6,6-DM, was also detected in fairly small amounts. This compound eluted from the charcoal column after maltose and had an R_G value of 1.33. Fractions from charcoal chromatography that contained 6-deoxy-oligosaccharides were pooled, and the modified products were further purified by descending paper chromatography (solvent A, 8-12 h). Larger oligosaccharides were not analyzed.

Product 6-DM was resolved into two peaks by l.c, which were designated 6-DM₁ and 6-DM₂. These were purified, then reduced with sodium borohydride, and hydrolyzed with acid to give the results shown in Figs. 1 and 2. Hydrolysis of reduced 6-DM₁ yielded 6-deoxy-D-glucose and D-glucitol in a 1:1 ratio, indicating that this compound is 6-deoxy-D-glucosyl- α -(1 \rightarrow 4)-D-glucose (6²-deoxymaltose). Similarly, reduced 6-DM₂ was hydrolyzed to give approximately equal amounts of 6-deoxy-D-glucitol and D-glucose, identifying this compound as D-glucosyl- α -(1 \rightarrow 4)-6-deoxy-D-glucose (6¹-deoxymaltose).

The products, 6-DG and 6,6-DM, were purified in amounts too small to be analyzed by this technique. Therefore, a more highly substituted amylose (d.s.=0.50) was hydrolyzed by PPA, and the products were separated and analyzed by the methods described previously. With these techniques, 6-DG and 6,6-DM were identified as 6-deoxy-D-glucose and 6-deoxy-D-glucosyl- α -(1,4)-6-deoxy-D-glucose (6¹,6²-di-deoxymaltose), as shown in Figs. 3 and 4.

Experiments using the more highly substituted 6-deoxyamylose also suggested that more 6²-deoxymaltose was formed than 6¹-deoxymaltose. Estimation from l.c. peak areas suggested that the ratio of 6²-deoxymaltose to 6¹-deoxymaltose was approximately 4:1. To examine this further, a sample of tritium-labeled 6-deoxyamylose (d.s.=0.15) was prepared by reduction of 6-deoxy-6-iodo-2,3,-di-O-acetylamylose with [³H]-sodium borohydride. Modified maltose was purified, reduced, and hydrolyzed, then separated by descending paper chromatography (solvent B, 6 h). The ratio

Figure 1. Analysis of 6-DM₁ by l.c. A). 6-DM₁. B). 6-DM₁ after reduction with sodium borohydride. C). Acid hydrolysis products of reduced 6-DM₁. (a), 6-deoxy-D-glucose; (b), D-glucitol. Conditions for chromatography are as described in the Experimental section.

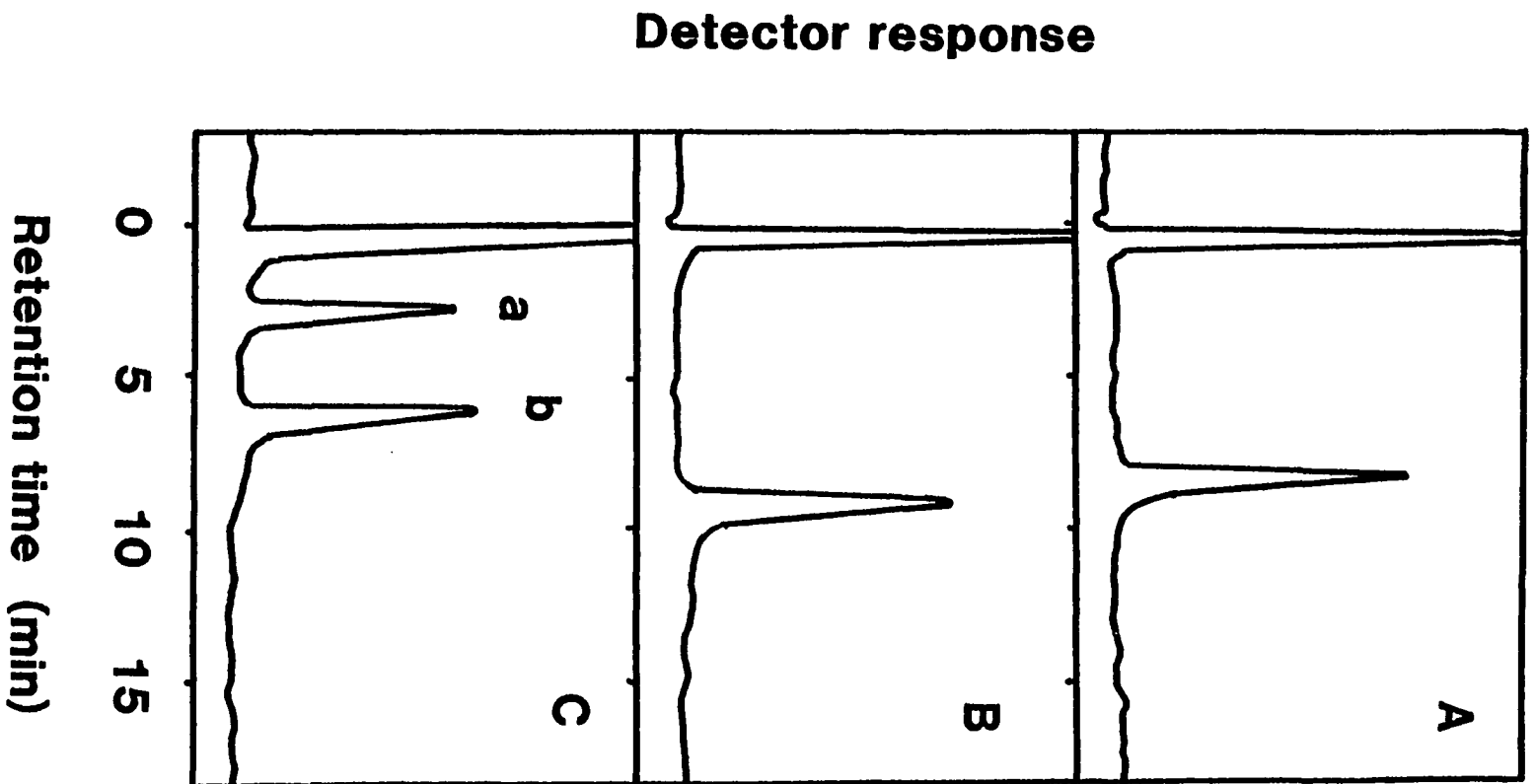


Figure 2. Analysis of 6-DM₂ by l.c. A). 6-DM₂. B). Reduced 6-DM₂.
C). Acid hydrolysis products of reduced 6-DM₂. (a), 6-deoxy-D-glucitol; (b), D-glucose.

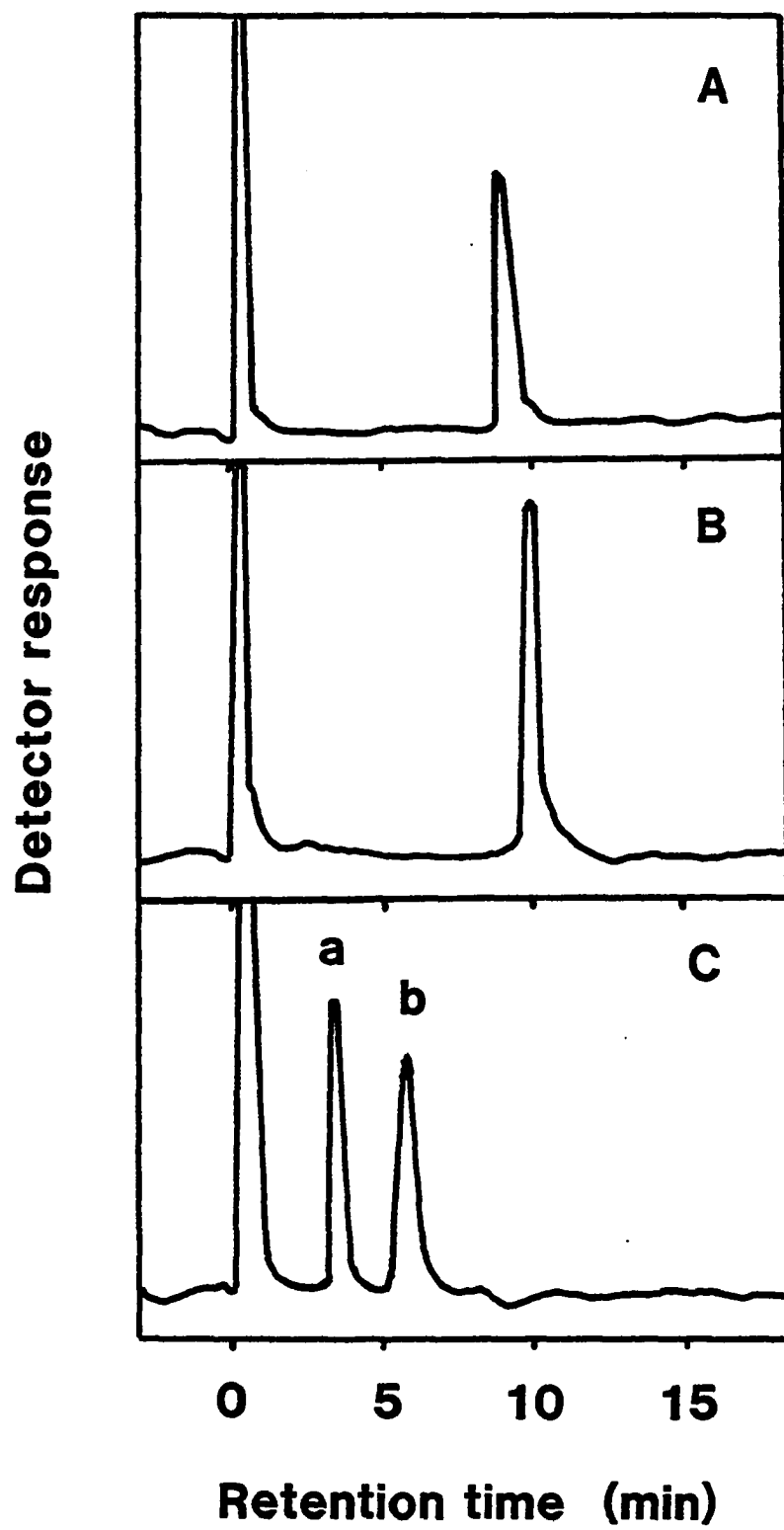


Figure 3. Analysis of 6-DG by l.c. A). 6-DG. (a), 6-deoxy-D-glucose. B). Reduced 6-DG. (a), 6-deoxy-D-glucitol. C). Acid hydrolysis products from reduced 6-DG. (a), 6-deoxy-D-glucitol.

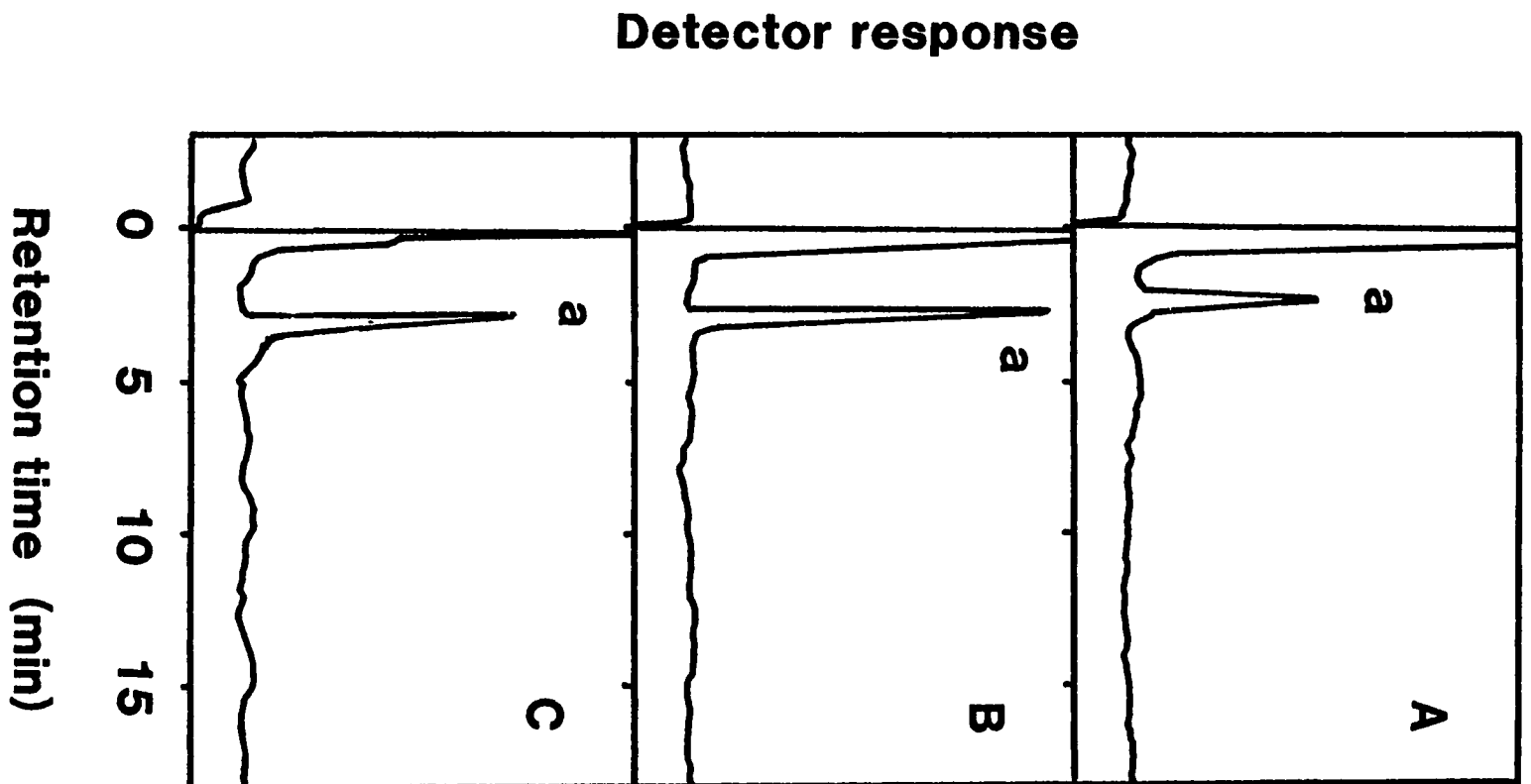
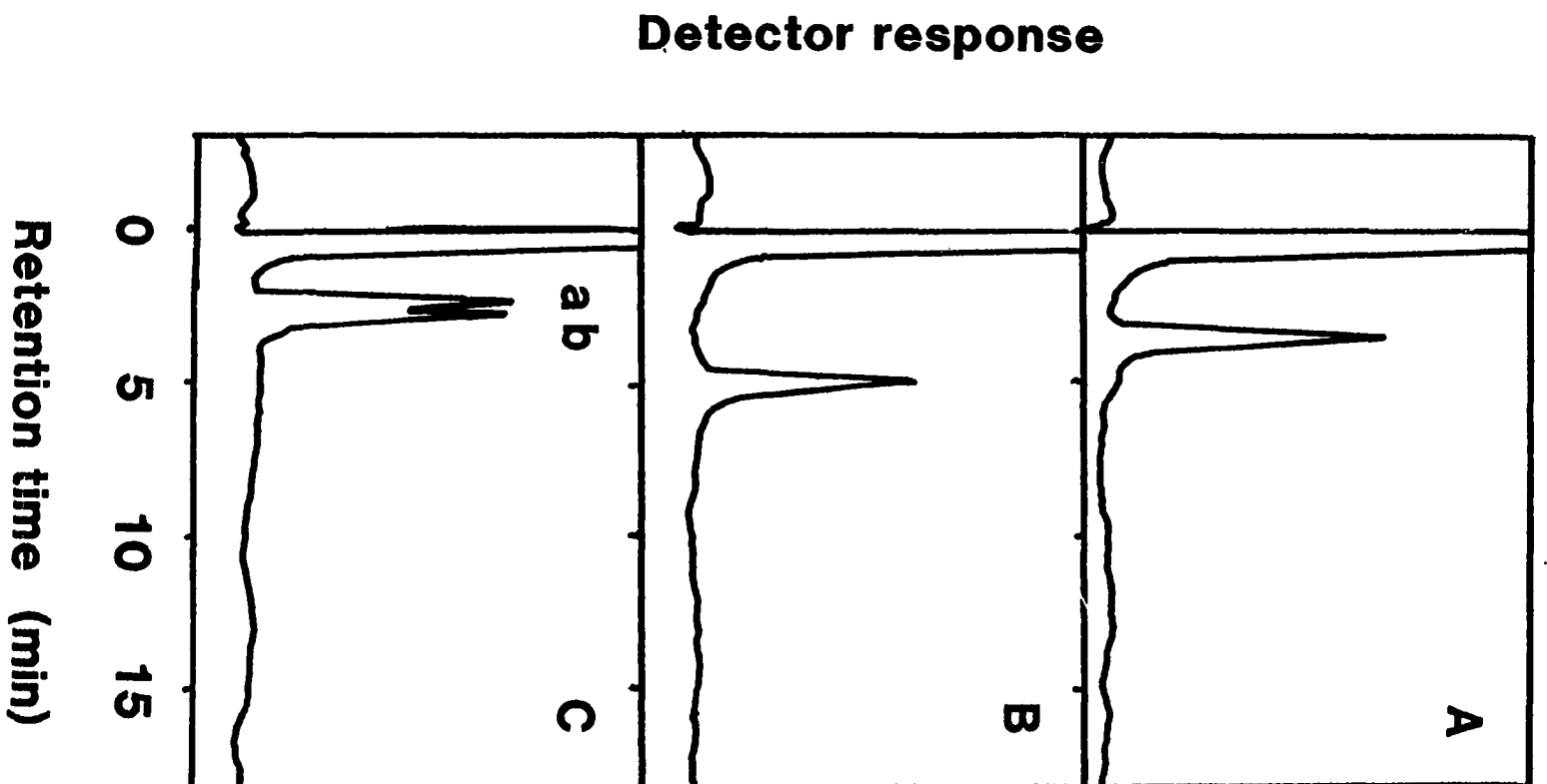


Figure 4. Analysis of 6,6-DM by l.c. A). 6,6-DM. B). Reduced 6,6-DM. C). Acid hydrolysis products of 6,6-DM. (a), 6-deoxy-D-glucose; (b), 6-deoxy-D-glucitol.



of radioactive 6-deoxy-D-glucose to 6-deoxy-D-glucitol was approximately 3:1, as determined by autoradiography and liquid scintillation.

PPA Hydrolysis of Mono-6-deoxy-6-fluoro- β -cyclodextrin

Because β -cyclodextrins are poor substrates for PPA and because of the low water solubility of the the fluorinated derivative, only small amounts of modified amylolysis products could be conveniently produced and analyzed. For this reason, products of PPA hydrolysis were reduced with [^3H]-sodium borohydride and analyzed by radiotracer techniques instead of methods used for analysis of 6-deoxysugars.

Paper chromatography (2 ascents, solvent A) of the final enzymolysis mixture showed the presence of small amounts of 6-deoxy-6-fluoro-D-glucose ($R_G = 1.19$). Paper chromatography of t-butyl alcohol fractions from charcoal chromatography showed 6-deoxy-6-fluoro-D-glucose eluting in the 1% t-butyl alcohol fraction (compared with D-glucose, which eluted with water). Another product appeared in the 2-3% t-butyl alcohol fractions. The product had paper chromatographic mobility similar to D-glucose ($R_G = 0.97$), but eluted from charcoal after maltose. A portion of each of the charcoal chromatography fractions, and of the final enzyme hydrolysis solution (100 μl each) was reduced with 1% sodium borohydride solution containing 0.2 mCi tritiated sodium borohydride. Descending paper chromatography (solvent B, 5 h) followed by autoradiography again showed two modified products. These components had mobilities relative to D-glucitol equal to 1.72 and 0.31, compared with values of 1.70, 1.00, and 0.11 for

6-deoxy-6-fluoro-D-glucitol, D-glucitol, and maltitol, respectively. On this basis, these compounds were identified as the reduced forms of 6-deoxy-6-fluoro-D-glucose and 6-deoxy-6-fluoromaltose.

The reduced 6-fluoromaltose mixture was purified by paper chromatography (solvent B, 22 h), and samples were hydrolyzed for either 2 or 4 h in 4.5 M TFA. Under these conditions, both samples contained less than 4% unhydrolyzed disaccharide. Paper chromatography (solvent B, 5 h descent) and autoradiography showed the presence of tritium-labeled 6-deoxy-6-fluoro-D-glucitol and D-glucitol. Results of liquid scintillation of these compounds estimated that the ratio of D-glucitol to 6-deoxy-6-fluoro-D-glucitol was 15:1 and 11:1 for the 2 h and 4 h hydrolysates, respectively. These results indicate that both 6-deoxy-6-fluoro-D-glucosyl- α -(1 \rightarrow 4)-D-glucose (6²-fluoromaltose) and D-glucosyl- α -(1 \rightarrow 4)-6-deoxy-6-fluoro-D-glucose (6¹-fluoromaltose) are produced during PPA hydrolysis but that the formation of the isomer modified in the nonreducing residue is greatly favored over the formation of the isomer modified in the reducing residue.

DISCUSSION

Substrates containing 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose gave similar results when hydrolyzed with porcine pancreatic α -amylase. In both instances, products included modified D-glucose and both possible isomers of maltose modified at C-6 of the D-glucose residues. In addition, for each of these substrates, formation of maltose modified in the nonreducing residue was preferred over formation of maltose modified in the reducing residue.

The active site of PPA has been shown to consist of five subsites, which bind a series of five D-glucose residues, with hydrolysis occurring between the second and third subsite from the reducing end (20). From analysis of the products obtained from amylolysis of modified substrates, it is possible to determine whether productive binding of a particular substitution occurs at individual subsites (Fig. 5). Several amylose substrates containing bulky modifications at C-6 have been observed previously (1-8). In no instances were modified D-glucose or maltose obtained as products of PPA hydrolysis. Substrate binding specificities for PPA substrates containing 6-O-hydroxyethyl-, 6-O-D-glucosyl-, and 6-phosphate groups are summarized in Table 1. In contrast to these modifications, which are not productively bound at subsites 3 or 4, both 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose may be productively bound at subsites 1 through 4. By comparison, it seems that bulky substituents such as hydroxyethyl groups and α -(1+6)-D-glucosyl branches affect enzyme-

Figure 5. Five subsite model for the active site of porcine pancreatic α -amylase, showing the formation of a modified disaccharide from a modified substrate. Formation of the modified product requires productive binding of the modified residue at subsites 2 and 4. (-O-), D-glucose; (- ϕ -), reducing residue; (- \bullet -), modified D-glucose. The point of catalytic attack is indicated by an arrow. The numbers 1-5 refer to individual subsites.

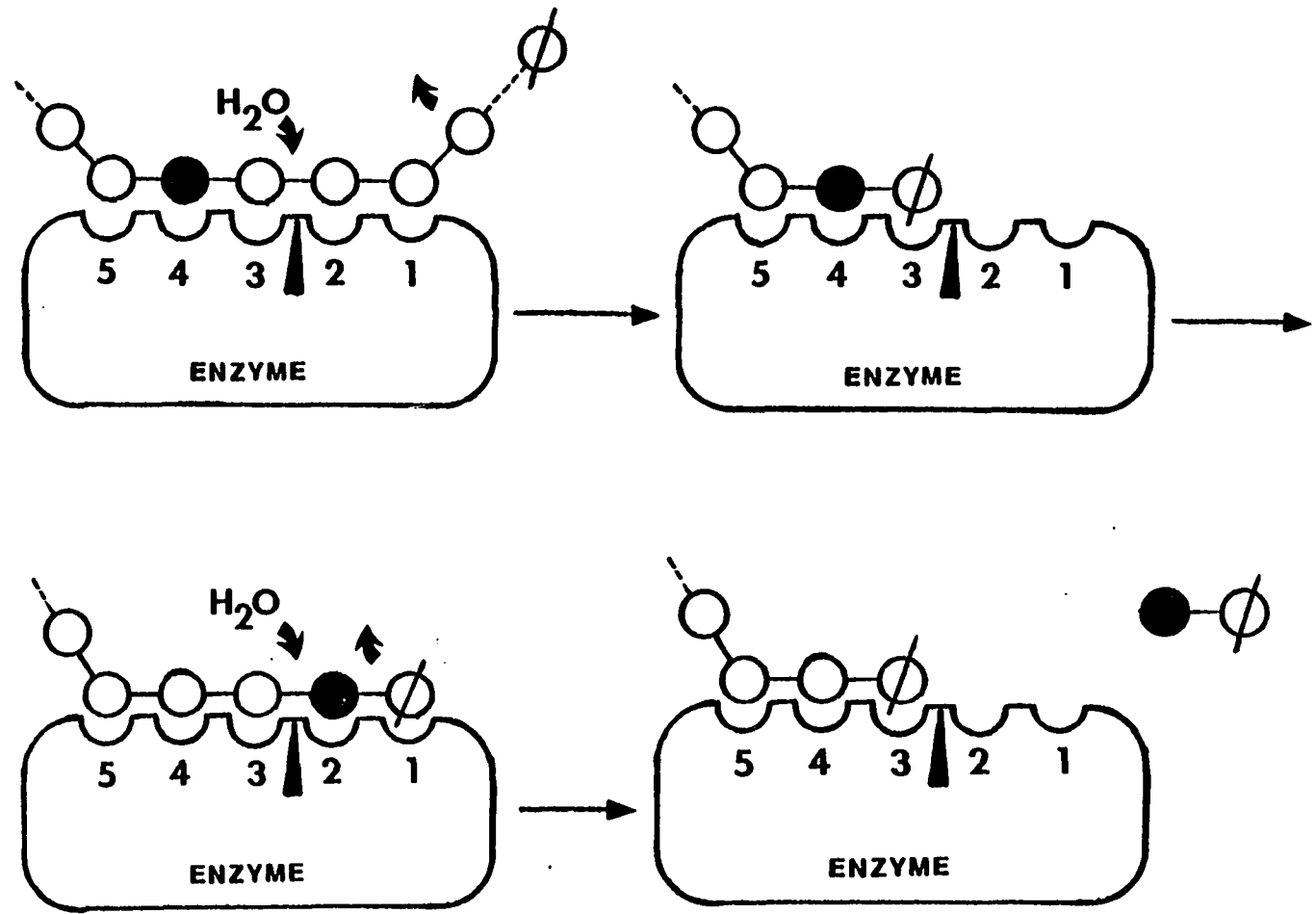


Table I. Productive Binding of Substrates Modified at C-6 to Subsites of Porcine Pancreatic α -Amylase

C-6 Modification	Porcine Pancreatic α -Amylase Subsite ^a					ref.
	1	2	3	4	5	
6-deoxy-	+	+	+ ^b	+	n.d.	this study
6-deoxy-6-fluoro-	+	+	+ ^b	+	n.d.	this study
6-O-hydroxyethyl-	+	+	-	-	+	8
6-O- α -D-glucosyl-	+	+	-	-	+	3
6-phosphate-	+	+	-	-	+	7

^aSubsite numbering as in Fig. 5. Plus signs indicate that productive binding is allowed. Minus signs indicate that productive binding is not allowed. N.d. indicates that binding has not been determined.

^bResults suggest that although productive binding at subsite 3 is allowed, it is not as favorable as binding of D-glucose.

substrate interactions mainly through steric disruption. Similar effects are also noted for the bulky, charged phosphate group.

Our results suggest that the deoxy- and deoxyfluoro- substituents are tolerated less well at the subsite where catalytic attack occurs (subsite 3) because formation of maltose modified in the reducing residue is less favored than formation of maltose modified in the nonreducing residue. Subsite 3 has been shown to be especially sensitive to substrate modification in experiments involving hydroxyethyl- (8) and 2-deoxy- derivatives (21). The ratio of disaccharides modified in the nonreducing residue to disaccharides modified in the reducing residue has also been shown to be greater for fluoromaltoses (11-15:1) than for deoxymaltoses (3-4:1). This difference may indicate that the 6-fluoro- group disrupts productive binding at subsite 3 to a greater extent than the 6-deoxy- modification.

Fluorine has often been considered to be a useful analog for hydroxyl groups, because it is about the same size, is highly electronegative, and contains non-bonded electron pairs similar to oxygen, which might allow it to act as a hydrogen bond acceptor (but not a hydrogen bond donor). Hydrogen bonding properties of fluorinated carbohydrates have not been thoroughly studied. Spectroscopic studies on compounds such as 2-fluoroethanol and 1-fluoro-2-propanol have shown that these molecules exist primarily in conformations favorable for intramolecular hydrogen bond formation (22-23). N.m.r. studies of Abraham and Monasterios (24) have shown, however, that the predominant rotational conformer for 2-fluoroethyl trichloroacetate is the same as that for 2-fluoroethanol, even though the added trichloroacetate ester would not be expected to partici-

pate in strong intramolecular hydrogen bonds. Further n.m.r. studies on 2-fluoroethanol by Griffith and Roberts (25) also gave no evidence for strong intramolecular hydrogen bond formation, and recent spectroscopic studies on 3-fluoro-1-propanol have concluded that hydrogen bonds in this compound must be weak compared with those of 3-amino-1-propanol (26). We have observed the behavior of 6-deoxy-6-fluoro-D-glucose on a variety of chromatographic media (l.c., t.l.c., paper chromatography, and charcoal chromatography). This compound consistently resembles 6-deoxy-D-glucose rather than D-glucose in its chromatographic mobility. Whether or not this observation is related to hydrogen bonding capabilities, it seems that fluorosugars do not closely resemble unmodified sugars with respect to their interactions with chromatographic media and solvents. Interactions with biomolecules, such as enzymes, may be similarly affected.

Other factors, associated with either substrate binding or hydrolysis, may contribute to the observed distribution of products as well. The rate of acid-catalyzed hydrolysis of α -methyl-6-deoxy-D-glucopyranoside is about 5 times greater than for α -methyl-D-glucopyranoside, which is, in turn, 6-7 times greater than those for the corresponding 6-halo-derivatives (27). We have observed, however, that glucosidic linkages of 2-deoxy-D-glucose residues, which are very acid-labile, are not readily hydrolyzed by PPA (21). Unfavorable interactions between enzyme and modified residues at subsites other than subsite 3 may also contribute to producing the observed product distributions. These might include less favorable interactions with 6-deoxy-D-glucose at subsites 2 or 4, or with 6-deoxy-6-fluoro-D-glucose at subsite 1. Alternatively, such results

might conceivably arise from unexpectedly favorable interactions between enzyme and modified substrate, such as highly favorable interactions with 6-deoxy-6-fluoro-D-glucose at subsite 2 or 4.

In any event, our results suggest that, although porcine pancreatic α -amylase activity is influenced by the presence of 6-deoxy-D-glucose or 6-deoxy-6-fluoro-D-glucose in the substrate, this effect is not crucial. The effects noted for larger modifying groups seem to originate largely from steric disruption, rather than from disruption of critical electronic or hydrogen bond interactions.

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SECTION II.

THE ACTION OF PORCINE PANCREATIC α -AMYLASE AND
Bacillus macerans CYCLODEXTRIN-GLUCANOTRANSFERASE
ON SUBSTRATES CONTAINING 2-DEOXY-D-GLUCOSE
AND 2-AMINO-2-DEOXY-D-GLUCOSE

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ABSTRACT

Modified glycogens containing radioactive 2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose have been synthesized using enzymatic techniques. Preferential synthesis of β -cyclodextrin containing single 2-deoxy-D-glucose residues was promoted by incubation of *Bacillus macerans* cyclodextrin-glucanotransferase with 2-deoxyglycogen in the presence of Triton X-100. Cyclic glucans containing 2-amino-2-deoxy-D-glucose were produced in a similar manner from glycogen containing 2-amino-2-deoxy-D-glucose. Hydrolysis of mono-2-deoxy- β -cyclodextrin with porcine pancreatic α -amylase (PPA) produced 2-deoxy-D-glucose, two isomers of 2-deoxymaltose, and a mixture of modified maltotrioses. These results indicate that 2-deoxy-D-glucose may be productively bound at all five subsites of the PPA active site. The distribution of the products suggests, however, that productive binding of the modified residue may not occur readily at the point of catalytic attack, and that the position of hydrolysis of modified substrates is different from that of unmodified substrates. Results of PPA hydrolysis of radiolabeled 2-amino-2-deoxyglycogen showed that a modified trisaccharide and a modified disaccharide were the smallest products formed by enzyme action. Analysis of these products by chemical and enzymatic techniques indicated that these products did not contain modified residues at their reducing termini. Formation of the observed 2-amino-2-deoxy-maltooligosaccharides is consistent with a scheme where productive binding of 2-amino-2-deoxy-D-glucose is allowed at subsites 1, 2, 4, and 5, but not at subsite 3, the subsite at which hydrolysis occurs.

INTRODUCTION

The active site of porcine pancreatic α -amylase (PPA) has been shown to consist of five subsites (1). Results of studies involving polysaccharides containing substituted hydroxyl groups have identified subsites which are sensitive to bulky modifications at C-2, C-3, and C-6 of the bound D-glucose residues (2-8). By comparison, relatively little work has been done examining the effect of charged modifications (9,10) or small uncharged substituents (11).

Glycogen containing 2-amino-2-deoxy-D-glucose has been previously shown to be depolymerized by PPA (9), but the products and kinetics of this reaction have not been investigated. Saccharides containing 2-deoxy-D-glucose have not been studied as PPA substrates, even though incorporation of 2-deoxy-D-glucose into glycogen using enzymatic techniques has been reported previously (12-14).

In order to fully examine the effect of modified D-glucose residues on the action of PPA, it is necessary that these residues be located in glycogen chain interiors, rather than only at chain termini. The modified glycogen containing 2-amino-2-deoxy-D-glucose used in these experiments was substituted at residues located throughout the chains. The glycogen preparation containing 2-deoxy-D-glucose was modified only at chain termini. To solve this problem, we attempted to prepare modified cyclodextrins. These dextrins are cyclic α -(1+4)-linked oligosaccharides synthesized by the action of bacterial enzymes on starch and related substrates (15). The most abundant of these compounds are α -cyclodextrin

and β -cyclodextrin which contain six and seven sugar residues per molecule, respectively. Although β -cyclodextrin is a relatively poor substrate for PPA, it is hydrolyzed much more readily than α -cyclodextrin. Therefore, we sought to promote the production of modified β -cyclodextrins by inclusion of phenolic detergents, which we have previously observed to be effective for this purpose (S. Kobayashi and D. French, Department of Biochemistry and Biophysics, Iowa State University, unpublished results).

In this report, we describe the synthesis of glycogen containing 2-deoxy-D-glucose by using uridine diphosphoglucose (UDPG)-glycogen glycosyl transferase from bovine heart, and the conversion of modified glycogens to cyclodextrins containing 2-deoxy- and 2-amino-2-deoxy-D-glucose, using the *Bacillus macerans* cyclodextrin-glucanotransferase. Mono-2-deoxy- β -cyclodextrin was hydrolyzed with PPA to obtain a number of modified oligosaccharides. These products have been analyzed, and the kinetics of their formation examined. The products from PPA hydrolysis of 2-amino-2-deoxy-glycogen have been isolated and analyzed in a similar manner. From the results of these experiments, it is possible to determine how these modifications affect binding at individual subsites of the PPA active site, and to identify features on the substrate which are crucial for binding and hydrolysis by PPA.

EXPERIMENTAL

Materials

Porcine pancreatic α -amylase (Boehringer-Mannheim Biochemicals, 10,000 U/mL), sweet potato β -amylase (Boehringer-Mannheim, 2,500 U/mL), and *Rhizopus niveus* glucoamylase (Seikagaku Fine Chemicals, 35 U/mg) were commercial materials and were used without further purification. The PPA contained no detectable α -glucosidase activity when assayed with p-nitro-phenyl- α -glucoside. Bovine heart UDPG-glycogen glycosyl transferase (2 U/mL) was provided by Dr. J. A. Thomas. Cyclodextrin-glucoamyltransferase from *B. macerans* was purified by the method of Kobayashi et al. (16) through the starch desorption step. Activity of this enzyme was assayed by using the method of Bender (17). Glycogen containing 2-amino-2-deoxy-D-glucose was a gift from Dr. W. J. Whelan. Shellfish glycogen was purchased from Sigma Chemical Co. Radioactive UDP-2-deoxy-D-glucose (20 μ Ci/mL, 0.4 mM) was purchased from NEN-DuPont, and the authenticity of this material was verified by hydrolysis in boiling water (1 h) followed by paper chromatography.

Methods

Paper chromatography was performed on Whatman 3MM paper, with 1-propanol:water, 7:3 (solvent A); 2-propanol:water:acetic acid, 27:9:4 (solvent B); or nitromethane:acetic acid:ethanol:saturated aqueous boric

acid, 8:1:1:1 (solvent C) used as solvents. Oligosaccharides containing 2-deoxy-D-glucose were hydrolyzed in sealed ampules for 1 h at 100° in either 2 M trifluoroacetic acid (TFA) to give total hydrolysis, or in 0.01 M TFA to hydrolyze 2-deoxy-D-glycosidic linkages. This mild acid treatment caused 90-95% release of 2-deoxy-D-glucose from 2-deoxyglycogen, and gave less than 5% hydrolysis of maltose. Reduction with sodium borohydride was performed by the method of Sloneker (18). Evaporations were performed under reduced pressure, at temperatures under 40°. Oligosaccharides containing 2-amino-2-deoxy-D-glucose were N-acetylated by a procedure similar to that of Roseman and Daffner (19). The modified oligosaccharides were treated with equal volumes of an ice-cold solution of acetic anhydride in water and saturated sodium bicarbonate. After 10 min at room temperature, the samples were placed in a boiling water bath for 3 min, then directly spotted on paper chromatograms or evaporated and used for further experiments. Monosaccharides on paper chromatograms were identified by comparison with authentic standards. Unlabeled aminosugar standards were detected with ninhydrin spray. Radioactivity of samples was determined by liquid scintillation in toluene cocktail using a Packard Model 2425.

Preparation of 2-Deoxyglycogen

Glycogen containing 2-deoxy-D-glucose was prepared by a procedure similar to that used by Biely et al. (12-13) for yeast enzymes. Radioactive UDP-2-deoxy-D-glucose (25 μ L) was dissolved in buffer solution (75

μL) containing 5 mM EDTA, 25 mM D-glucose-6-phosphate, 100 mM Tris-HCl and 10% glycogen, at a pH of 7.8. Two aliquots (2 μL) were removed, spotted on paper squares (1.5 x 1.5 cm), and dried. Both were washed for 20 min in cold 100% ethanol, then one of the samples was washed in hot 66% ethanol. These samples provided values for total radioactivity and for background radioactivity. Bovine heart UDPG-glycogen glycosyl transferase was preincubated in 5 mM dithiothreitol at 30° for 20 min. Enzyme solution (4 μL) was added to the substrate, and the mixture was incubated at 30°. Samples (2 μL) were removed at 1, 2, 3, 4, 5, and 24 h, spotted on paper squares, and dried. The squares were then washed successively in cold 100% ethanol and hot 66% ethanol. Incorporation of radioactivity into polysaccharide was measured by liquid scintillation of the dried squares.

Treatment of Modified Glycogens with Cyclodextrin-Glucanotransferase

Labeled 2-deoxyglycogen was treated with cyclodextrin-glucanotransferase by preparing two enzyme reaction mixtures. These consisted of a 2% 2-deoxyglycogen solution (400 μL), 0.1 M calcium acetate buffer, pH 6.0, containing 1 mM calcium chloride and *B. macerans* cyclodextrin-glucanotransferase (0.66 U/mL, 200 μL), and either water or 4% Triton X-100 (200 μL). Both mixtures were incubated at 37°. Samples (50 μL) were removed at 1, 8, and 24 h, and spotted on paper chromatograms. Paper chromatography (2 ascents, solvent A) and autoradiography were then performed. Modified 2-deoxy-β-cyclodextrin for further experiments was prepared by

scaling-up the cyclodextrin-glucanotransferase reaction mixture 10-fold and allowing the reaction to proceed for 16-20 h.

Preparations containing a 10% solution of 2-amino-2-deoxyglycogen (150 μ L), *B. macerans* cyclodextrin-glucanotransferase in acetate buffer (1.6 U/mL, 75 μ L), and either water or Triton X-100 (75 μ L) were incubated at 37°. Samples (50 μ L) were removed at 2, 6, 24, and 48 h, and were acetylated by addition of 5% acetic anhydride and sodium bicarbonate (15 μ L each) as described in the Methods section. Products of this reaction were then examined by paper chromatography (solvent B, 2 descents, 8 h each) and autoradiography. A parallel experiment using 2-deoxyglycogen was also performed.

Digestion of Modified Saccharides with PPA

A solution of mono-2-deoxy- β -cyclodextrin (150 μ L, 120,000 dpm) water (100 μ L), and 50 μ L buffer, pH 6.9, containing 200 mM sodium glycerophosphate buffer, 100 mM sodium chloride, and 0.2% sodium azide was prepared. Enzyme solution was prepared by centrifuging PPA (100 μ L) and dissolving the pellet in buffer (200 μ L) containing 20 mM sodium glycerophosphate, 10 mM calcium chloride, and 0.02% sodium azide, pH 6.9. Reaction was started by the addition of 200 μ L of PPA solution to 300 μ L of substrate. The mixture was incubated at 37°. Aliquots (25 μ L) were removed at 4, 8, 24, 48, and 96 h, and spotted on two identical paper chromatograms. These chromatograms were ascended twice (solvent A) and autoradiographed.

A 10% solution of 2-amino-2-deoxyglycogen (200 μ L) and 20 μ L of buffer, pH 6.9, containing 200 mM sodium glycerophosphate, 100 mM sodium chloride, and 0.2% sodium azide buffer was prepared. A solution of PPA (30 μ L) and buffer, pH 6.9, containing 20 mM sodium glycerophosphate, 10 mM calcium chloride, 0.02% sodium azide buffer (30 μ L), pH 6.9, was added to the substrate. The solution was incubated at 37^o, and aliquots (35 μ L) were removed at 1, 4, 8, and 24 hrs. These samples, and a sample of 10% 2-aminoglycogen were acetylated by addition of 5% aqueous acetic anhydride and saturated sodium bicarbonate (15 μ L each) as described previously. The samples were then spotted on paper and separated (descending chromatography, 8 h, solvent B).

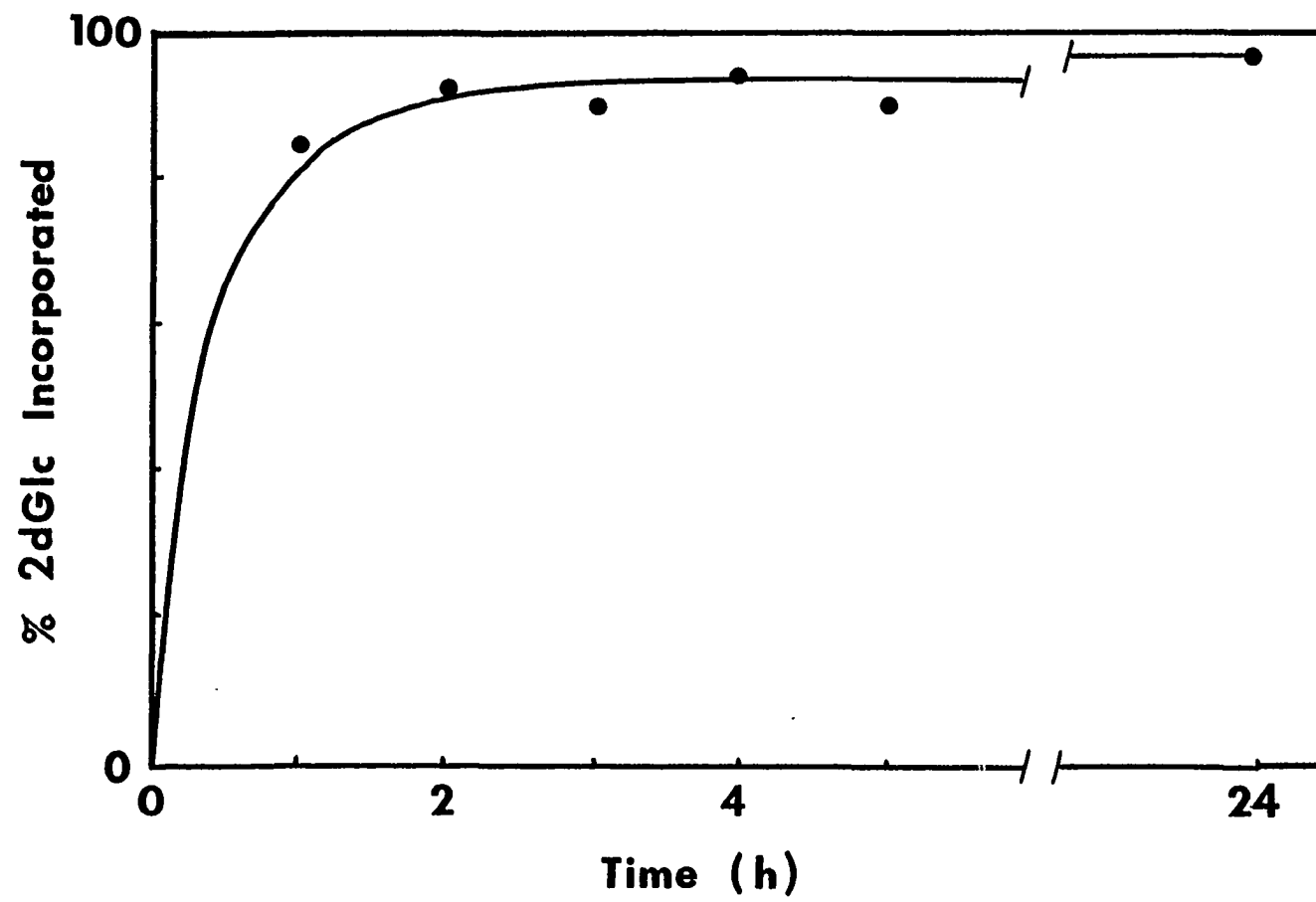
RESULTS

Incorporation of radioactive 2-deoxy-D-glucose into glycogen using bovine heart UDPG-glycogen glycosyl transferase was rapid and essentially complete under the conditions of the experiment (Fig. 1). Identical results were obtained when this reaction was repeated on a 10-fold larger scale. The polysaccharide product of the large scale preparation was isolated by precipitation with several volumes of cold 100% ethanol and centrifugation, and the precipitate was washed with 65% ethanol.

Treatment of this polysaccharide with β -amylase resulted in formation of a single modified disaccharide product (2-deoxy-D-glucosyl- α -(1+4)-D-glucose), rather than the two products observed by Biely et al. (12,13). This indicates that only single 2-deoxy-D-glucose residues are introduced at chain termini under these conditions. This may result either from the use of radiotracer amounts of modified sugar nucleotide in this experiment, or from a difference in specificity between bovine and yeast enzymes. Digestion of 2-deoxyglycogen with PPA gave this same disaccharide as a product.

Incubation of this sparsely modified glycogen with excess UDPG and bovine glycosyl transferase proved not to be an efficient method for adding D-glucose to the nonreducing termini of modified chains (data not shown). Mild acid hydrolysis of the product from this reaction caused release of less than 4% the total radioactivity in the form of 2-deoxy-oligosaccharides. Since small amounts of these oligosaccharides may arise from cleavage of D-glucosidic, rather than 2-deoxy-D-glucosidic

Figure 1. Incorporation of radioactive 2-deoxy-D-glucose (2dGlc) into glycogen using bovine heart UDPG-glycogen glycosyltransferase.

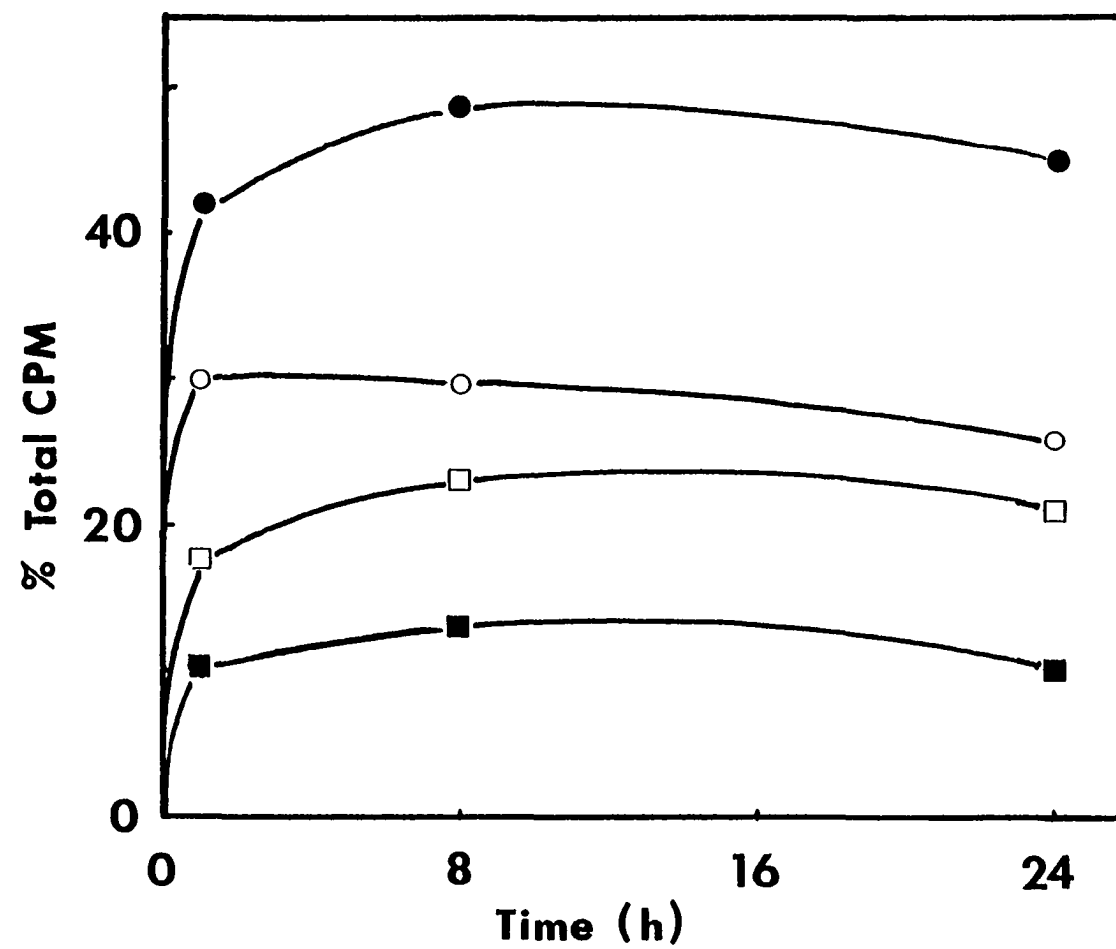


bonds, these results suggest that D-glucose is added exclusively to unmodified chain ends.

The action of cyclodextrin-glucanotransferase on 2-deoxyglycogen is shown on Fig. 2. Two principal products were formed in this reaction, with paper chromatographic mobilities corresponding to those predicted for mono-2-deoxy- α -cyclodextrin and - β -cyclodextrin (20). Formation of 2-deoxy- β -cyclodextrin could be preferentially promoted by inclusion of Triton X-100 in the enzyme solution, consistent with earlier observations in our laboratory (Kobayashi and French, unpublished results). In addition to promoting the formation of β -cyclodextrins, the presence of Triton X-100 was also found to accelerate the depolymerization of the modified glycogen. Modified cyclodextrins were separated by paper chromatography (2 ascents, solvent A). Linear oligosaccharide products were removed from these fractions by incubation with β -amylase (250 U/mL in 50 mM sodium acetate, pH 4.8). Results of these experiments indicated that the 2-deoxy- β -cyclodextrin fraction contained less than 5% radioactive product which was susceptible to β -amylase action, whereas the crude 2-deoxy- α -cyclodextrin fraction contained substantial amounts of non-cyclic products. The linear component, which comprised as much as 60-65% of the radioactivity in the crude 2-deoxy- α -cyclodextrin fraction, was rapidly hydrolyzed by β -amylase, leaving modified cyclodextrin which was resistant to further β -amylase hydrolysis.

Treatment of 2-deoxy- β -cyclodextrin with PPA gave rise to four major products which were tentatively identified as 2-deoxy-D-glucose, two isomers of 2-deoxymaltose, and an unresolved mixture of 2-deoxymaltotriose

Figure 2. Formation of modified cyclodextrins from 2-deoxyglycogen in presence and absence of Triton X-100. (-●-), 2-deoxy- β -cyclodextrin, Triton X-100 added; (-○-), 2-deoxy- β -cyclodextrin, no Triton X-100; (-■-), 2-deoxy- α -cyclodextrin, Triton X-100 added; (-□-), 2-deoxy- α -cyclodextrin, no Triton X-100.



isomers (Fig. 3). These products were numbered 1-4 in order of decreasing chromatographic mobility. Product 1 was identified as 2-deoxy-D-glucose on the basis of its paper chromatographic mobility. Product 3 had the same mobility as a product obtained from hydrolysis of nonreducing end-modified 2-deoxyglycogen with either PPA or sweet potato β -amylase. Mild acid hydrolysis of this material released 2-deoxy-D-glucose (lane 3-A in Fig. 3). Based on these results, the structure of product 3 has been assigned as 2-deoxy-D-glucosyl- α -(1 \rightarrow 4)-D-glucose (2²-deoxymaltose). Product 2 (lane 2) had paper chromatographic mobility slightly greater than that of product 3, but less than that of 2-deoxy-D-glucose. Mild acid hydrolysis did not affect product 2 (lane 2-A), and reduction of product 2 with sodium borohydride followed by total acid hydrolysis produced radioactive 2-deoxy-D-glucitol (data not shown). These results identified product 2 as D-glucosyl- α -(1 \rightarrow 4)-2-deoxy-D-glucose (2¹-deoxymaltose). Mild acid hydrolysis of product 4 gave an unequal mixture of 2-deoxy-D-glucose, 2¹-deoxymaltose, and a modified maltotriose containing 2-deoxy-D-glucose in the reducing residue (2¹-deoxymaltotriose).

Kinetics of hydrolysis of 2-deoxy- β -cyclodextrin are shown in Fig. 4a. A mixture of modified trisaccharides is rapidly produced, then slowly hydrolyzed under these conditions. The principal product of extensive PPA hydrolysis is 2²-deoxymaltose, with considerably smaller amounts of 2¹-deoxymaltose and 2-deoxy-D-glucose.

Two identical chromatograms were prepared in obtaining the results shown in Fig. 4. The spots containing 2-deoxymaltotriose were cut out and eluted from the second chromatogram. Radioactive trisaccharides were

Figure 3. Products of hydrolysis of mono-2-deoxy- β -cyclodextrin with PPA. Std, maltooligosaccharide standards, G_1 - G_n ; DS, disaccharide product from hydrolysis of 2-deoxyglycogen with β -amylase; 1, product 1 from PPA hydrolysis of mono-2-deoxy- β -cyclodextrin (2-deoxy-D-glucose); 2, product 2 (2^1 -deoxymaltose); 2-A, product 2 after mild acid hydrolysis; 3, product 3 (2^2 -deoxymaltose); 3-A, product 3 after mild acid hydrolysis; 4, product 4; 4-A, product 4 after mild acid hydrolysis.

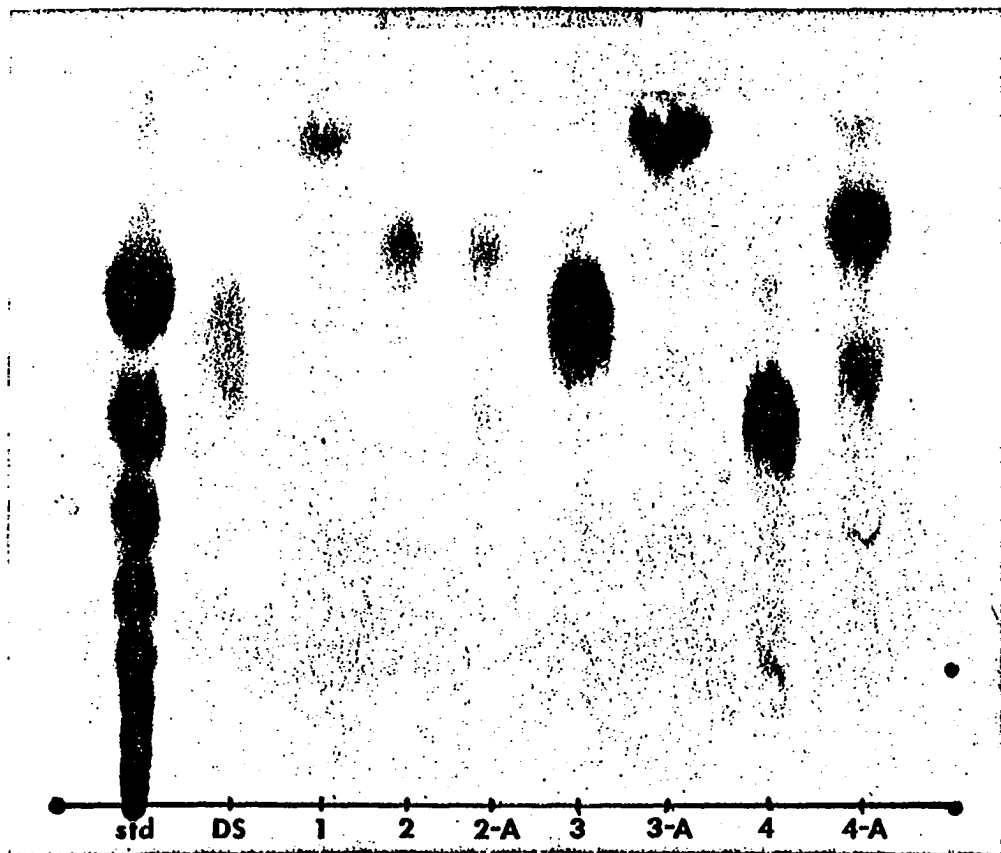


Figure 4a. Kinetics of PPA hydrolysis of mono-2-deoxy- β -cyclodextrin.

(-●-), 2-deoxymaltotrioses; (-○-), 2²-deoxymaltose;

(-■-), 2¹-deoxymaltose; (-□-), 2-deoxy-D-glucose.

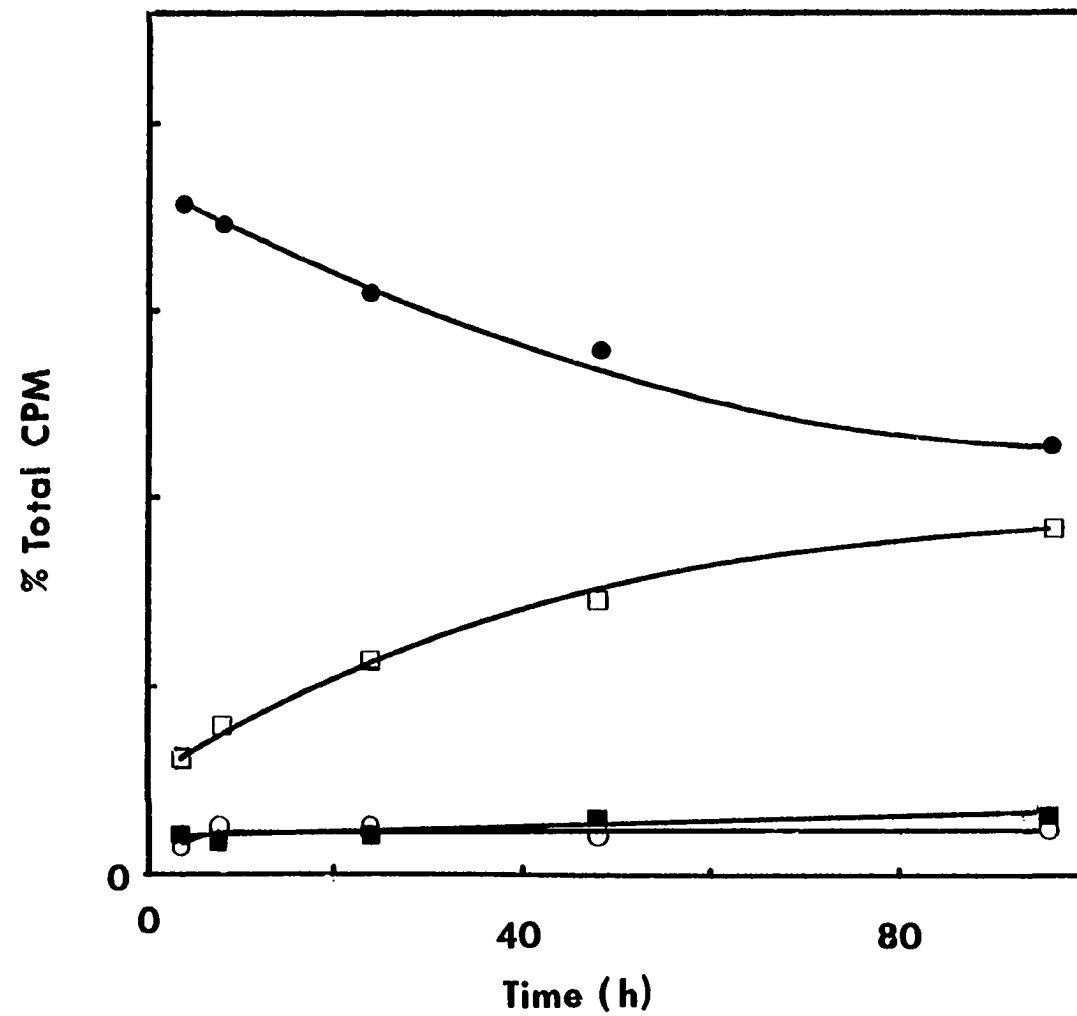
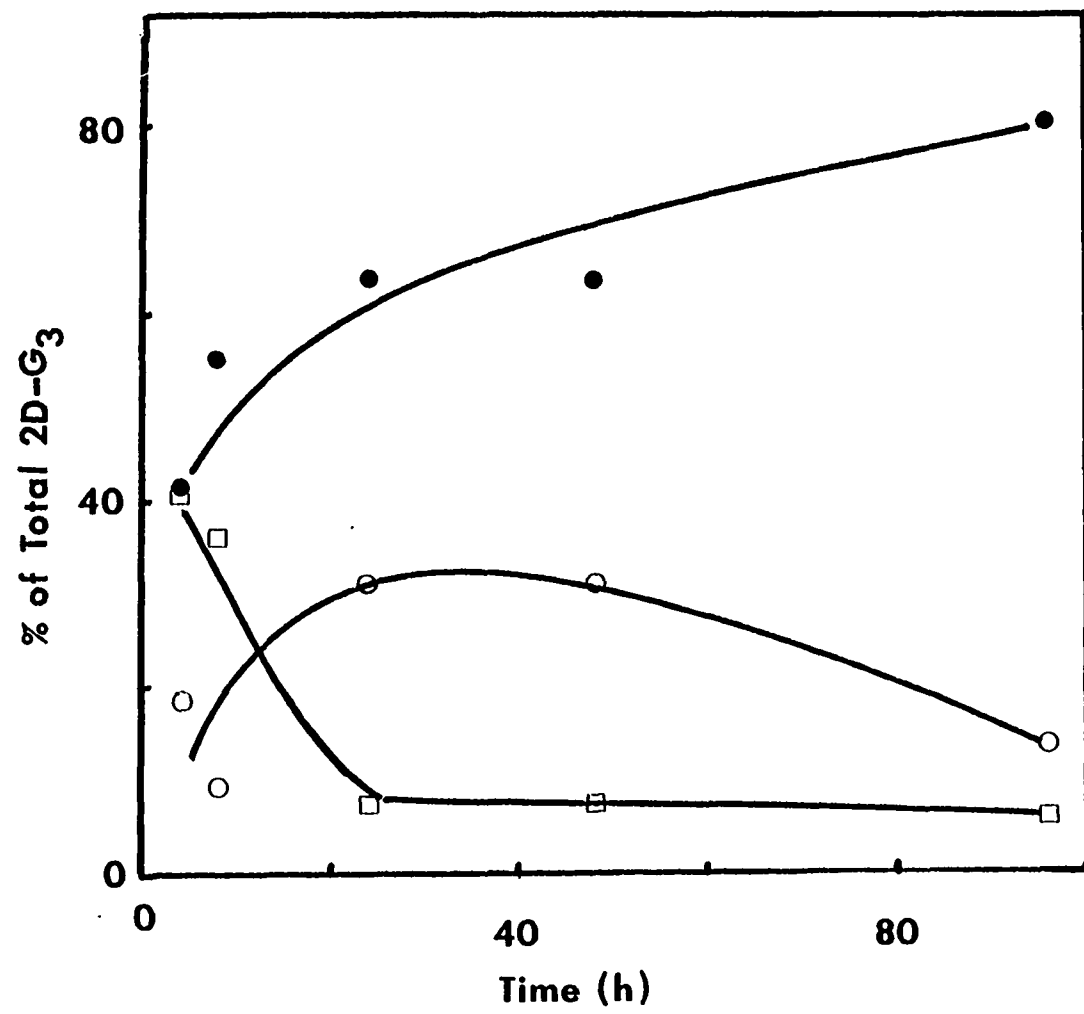


Figure 4b. Change in composition of 2-deoxymaltotriose (2D-G₃) fraction during PPA hydrolysis. (-○-), 2¹-deoxymaltotriose; (-●-), 2²-deoxymaltotriose; (-□-), 2³-deoxymaltotriose.



eluted from the paper, evaporated, subjected to mild acid hydrolysis, and rechromatographed. Results of this experiment (Fig. 4b) showed that the composition of the deoxymaltotriose fraction changes considerably during the course of the reaction. Since less than 10% of the total original radioactivity is found in the form of products larger than trisaccharides after 4 h of reaction, these changes in composition arise mainly from different relative rates of hydrolysis of the 2-deoxy-trisaccharides. These results indicate that 2³-deoxymaltotriose is rapidly cleaved, with slower rates of PPA hydrolysis for 2¹-deoxymaltotriose and 2²-deoxymaltotriose. Similar experiments were performed using a mixture of purified 2-deoxymaltotrioses as substrate for PPA (data not shown). Results of this latter experiment also indicated the same trend in relative rates of hydrolysis.

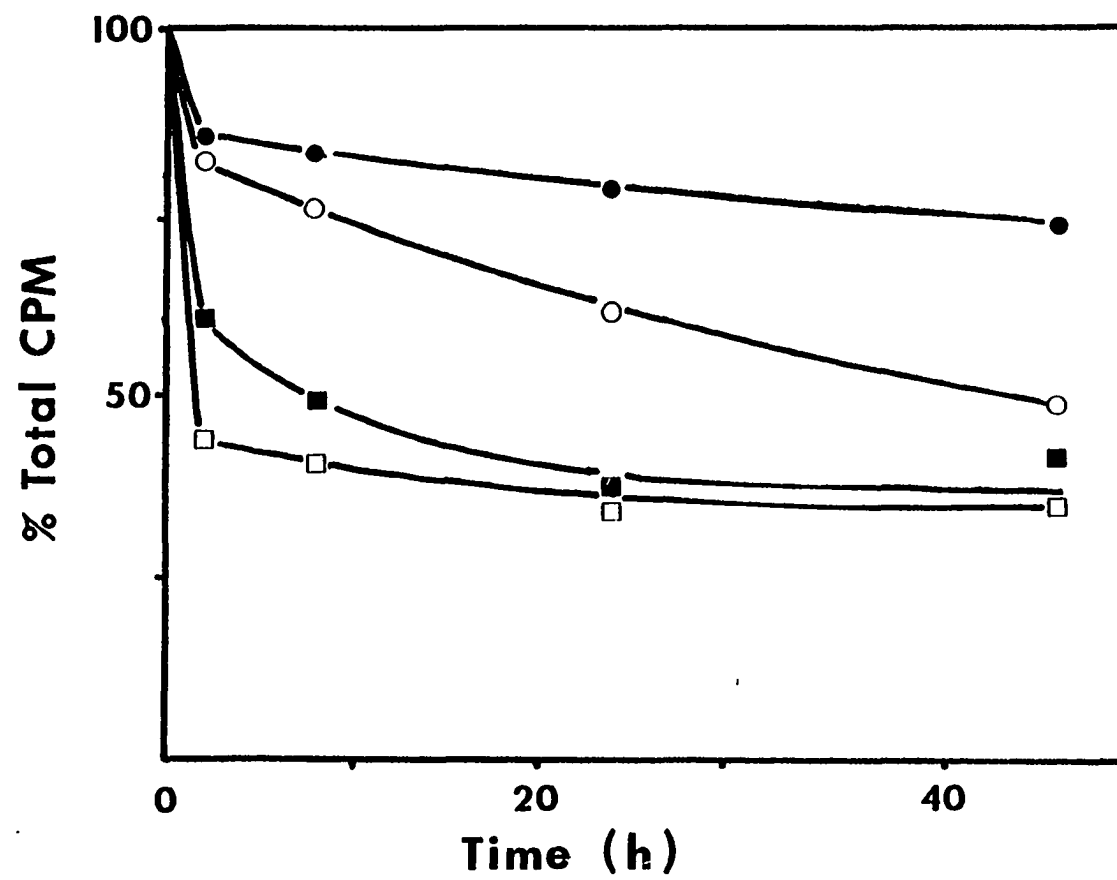
These experiments suggested that the glycosidic bond which is hydrolyzed by PPA may differ for different deoxymaltotriose isomers. Hydrolysis of unmodified maltotriose by PPA results in 85% cleavage of the glucosidic bond between the first and second residues from the reducing end, and 15% cleavage of the glucosidic bond between the second and third residues (1). Because of limitations in the mild acid hydrolysis technique, we were unable to calculate exact bond-cleavage frequencies for deoxymaltotrioses. However, in order to account for the large amount of 2²-deoxymaltose formed, the major (or only) point of hydrolysis of 2³-deoxymaltotriose must be between the first and second residues from the reducing end, whereas hydrolysis of 2²-deoxymaltotriose must occur largely (or exclusively) between the second and third residues. The third isomer,

2¹-deoxymaltotriose, seems to be hydrolyzed at either of the glucosidic bonds, giving rise to most of the 2-deoxy-D-glucose and 2¹-deoxymaltose formed in the enzyme reaction. Conclusions about the point of cleavage of 2³-deoxymaltotriose are supported by our observation that after extensive PPA hydrolysis of nonreducing-end modified 2-deoxyglycogen, 2²-deoxymaltose is the exclusive product, and no 2-deoxy-D-glucose is formed.

When 2-amino-2-deoxyglycogen was treated with cyclodextrin-glucanotransferase, conversion of polysaccharide to lower-molecular-weight species was relatively slower than for 2-deoxyglycogen (Fig. 5). This difference might be partly accounted for by the structures of the modified glycogens. The 2-deoxy-D-glucose residues in 2-deoxyglycogen were located at the nonreducing termini of the chains, whereas 2-amino-2-deoxy-D-glucose residues were distributed throughout the modified glycogen. Differences might also arise from other structural features, such as differences in the degree of branching or the location of modified residues with respect to branch points. Action of the cyclodextrin-glucanotransferase may also be seriously affected by the presence of amino groups in the substrate.

Presence of Triton X-100 in the cyclodextrin enzyme reaction mixture accelerated the depolymerization of both modified glycogens, as seen in Fig. 5. Since depolymerization of 2-deoxyglycogen approaches the same limiting value in either the presence or absence of detergent, this surfactant seems to be increasing the rate of depolymerization without affecting the susceptibility of resistant portions of the glycogen molecule. The products obtained from treatment of 2-amino-2-deoxyglycogen with

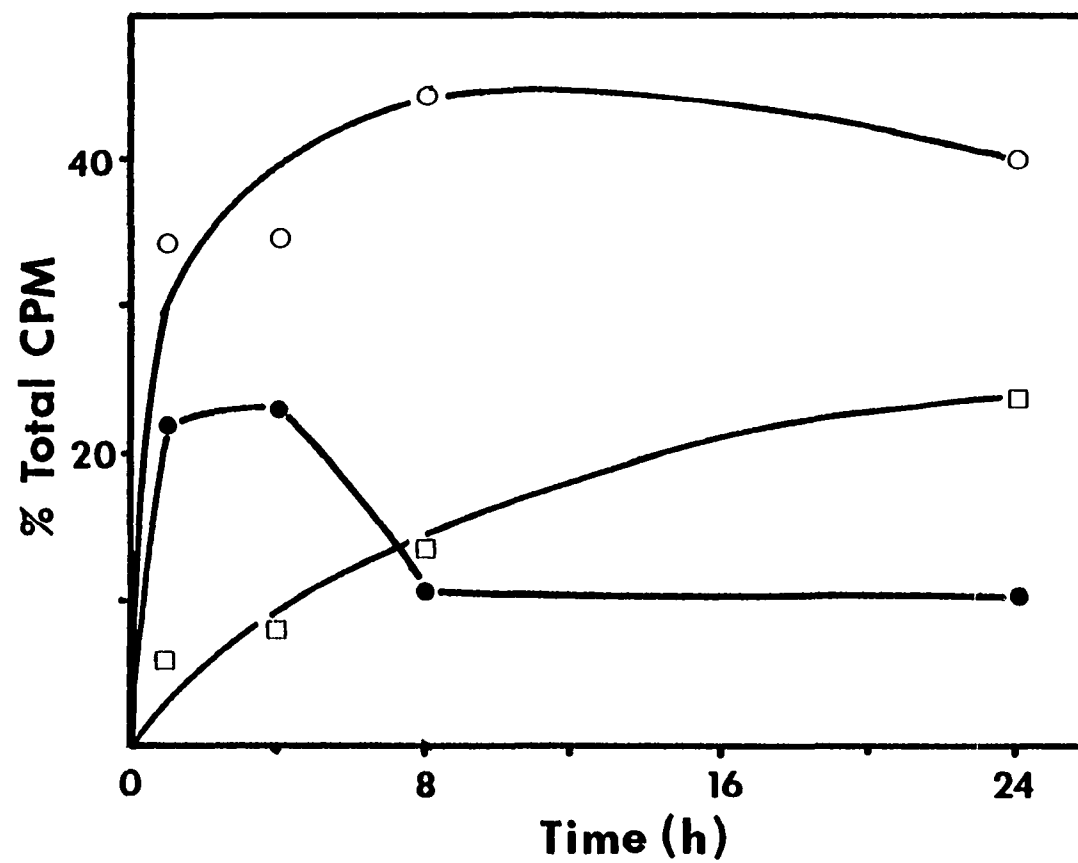
Figure 5. Depolymerization of modified glycogens in presence and absence of Triton X-100. This figure shows the fraction of original radioactive polysaccharide which has not been converted to cyclodextrins and small oligosaccharides (linear hexasaccharides and smaller). (-●-), 2-amino-glycogen, no Triton X-100; (-○-), 2-aminoglcogen, Triton X-100 added; (-■-), 2-deoxyglycogen, no Triton X-100; (-□-), 2-deoxyglycogen, Triton X-100 added.



cyclodextrin-glucanotransferase were N-acetylated and separated by paper chromatography. These products appeared as a poorly resolved mixture of oligosaccharides after detection by autoradiography. Products having chromatographic mobilities predicted for N-acetylated aminocyclodextrins were obtained. This fraction contained a component that was resistant to prolonged hydrolysis with a combination of β -amylase and glucoamylase. A second reaction was performed, in which the products obtained from digestion of 2-aminoglycogen with cyclodextrin-glucanotransferase were not N-acetylated prior to paper chromatography. A modified oligosaccharide fraction was produced in this reaction which was also resistant to hydrolysis by β -amylase.

PPA hydrolysis of 2-amino-2-deoxyglycogen gave rise to three major low-molecular-weight products. The N-acetylated derivatives of these products had chromatographic mobilities corresponding to those predicted for N-acetylated 2-amino-2-deoxy-tetrasaccharide, -trisaccharide, and -disaccharide products. The N-acetylated disaccharide had the same chromatographic mobility as a product obtained when 2-amino-2-deoxyglycogen was hydrolyzed with β -amylase, then N-acetylated. Kinetics of this reaction (Fig. 6) show that the modified tetrasaccharide and trisaccharide products are rapidly formed, then subsequently hydrolyzed to form the disaccharide. These results are consistent with those of Maley *et al.* (9,21), who observed two products from PPA hydrolysis of 2-amino-2-deoxyglycogen, which seem from their data to be tri- and tetra-saccharides, as well as very small amounts of a product migrating with β -amylase product.

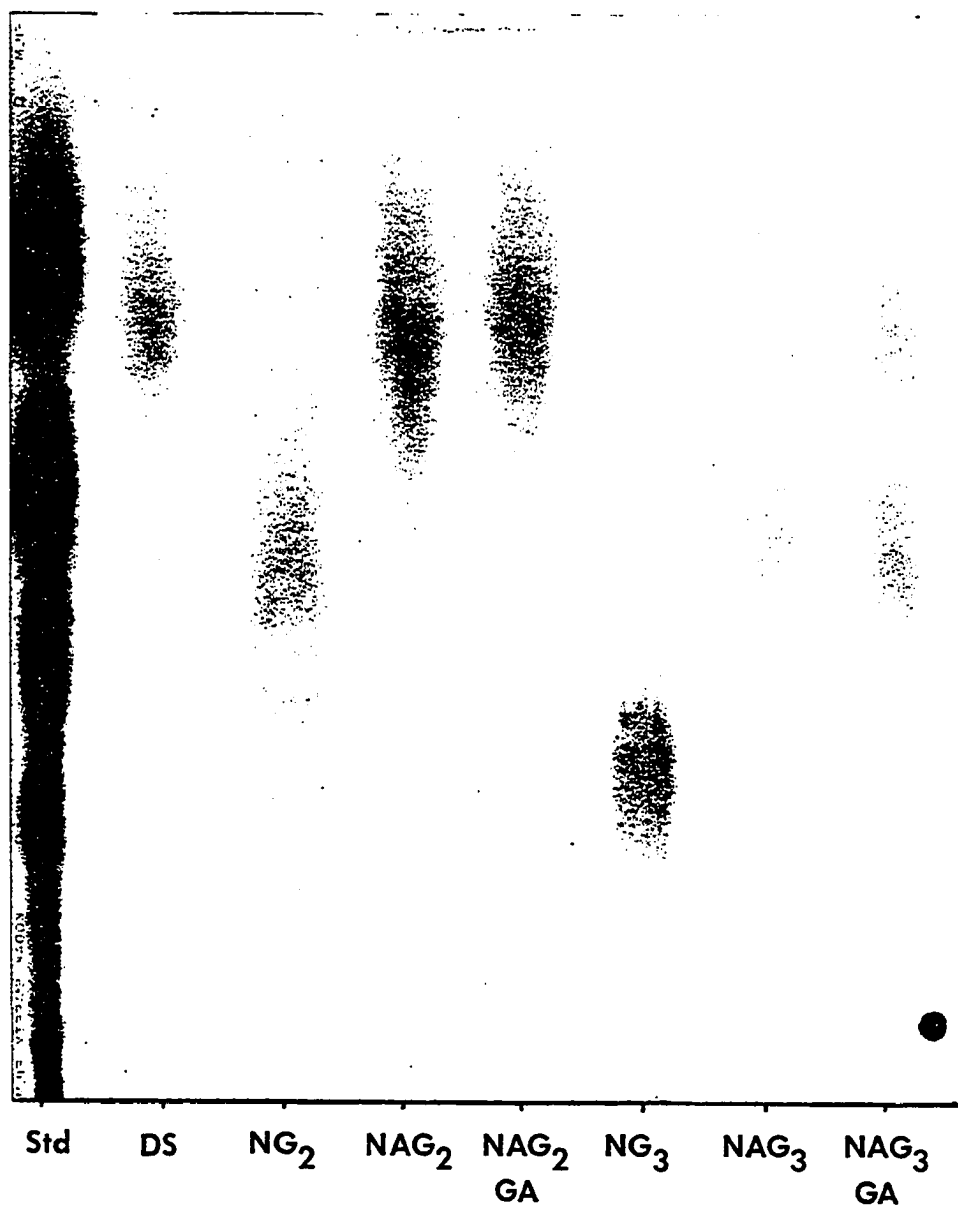
Figure 6. Kinetics of digestion of 2-aminoglycogen with PPA. (-●-), 2-amino-tetrasaccharide; (-○-), 2-amino-trisaccharide; (-□-), 2-amino-disaccharide.



In contrast to the results obtained for PPA hydrolysis of 2-deoxyglycogen, no modified monosaccharide is liberated.

To further characterize these PPA hydrolysis products, the N-acetylated disaccharide and trisaccharide products were treated with glucoamylase. It has been observed by Romero et al. (22), and confirmed in this laboratory, that glucoamylase releases 2-amino-2-deoxy-D-glucose from modified glycogen. We have also observed that *R. niveus* glucoamylase action is blocked by the presence of a bulky substituent at C-2, but that glucoamylase will hydrolyze the adjacent glucosidic linkage on the non-reducing side of the modified residue. Samples of N-acetylamino-maltose and -maltotriose dissolved in water (100 μ L), were mixed with 50 mM sodium acetate buffer, pH 4.8 (50 μ L) and glucoamylase (35 U/mL, 50 μ L). The solutions were incubated at 37 $^{\circ}$, and aliquots (80 μ L) were removed at 5 and 24 h. These were separated by paper chromatography, as in previous experiments. Results (Fig. 7) show that the N-acetylamino-disaccharide is not hydrolyzed by glucoamylase, suggesting that the structure of the original disaccharide is 2-amino-2-deoxy-D-glucosyl- α -(1 \rightarrow 4)-D-glucose (2 2 -aminomaltose). The N-acetylamino-trisaccharide is partially hydrolyzed by glucoamylase to produce a modified disaccharide that is resistant to further hydrolysis. This suggests that the trisaccharide fraction contains components modified in the second and third residues from the reducing end (2 2 -amino- and 2 3 -amino-maltotriose, respectively). A mixture of the disaccharide and trisaccharide products was reduced with sodium borohydride, then hydrolyzed with 3M TFA for 4 h at 100 $^{\circ}$ under nitrogen. Paper chromatography (solvent C, 6 h descent) and autoradiography showed

Figure 7. Analysis of 2-amino-oligosaccharides from PPA hydrolysis of 2-aminoglycogen. Std, maltooligosaccharide standards, G_1 - G_n ; DS, N-acetylated product from β -amylolysis of 2-aminoglycogen; NG_2 , 2-amino-disaccharide product from PPA hydrolysis of 2-aminoglycogen; NAG_2 , N-acetylated NG_2 ; NAG_2 -GA, NAG_2 after treatment with *R. niveus* glucoamylase; NG_3 , 2-amino-trisaccharide; NAG_3 , N-acetylated 2-amino-trisaccharide; NAG_3 -GA, NAG_3 after glucoamylase treatment.



that 2-amino-2-deoxy-D-glucose was the only major product detected, although hydrolysis was not complete and some acid degradation occurred. On the basis of results obtained from glucoamylase digestion and acid hydrolysis experiments, we concluded that 2-amino-oligosaccharides containing modified residues in the reducing end are not produced during PPA hydrolysis. As noted previously, 2-amino-2-deoxy-D-glucose is not produced by PPA hydrolysis of 2-aminoglycogen.

DISCUSSION

Modified glycogens may be produced by the action of appropriate glycogen-synthesizing enzymes on the UDP derivatives of 2-deoxy-D-glucose (12-14, this study) and 2-amino-2-deoxy-D-glucose (9,21,22). These modified glycogens may be further converted to cyclodextrins by *B. macerans* cyclodextrin-glucanotransferase. Either the glycogens or cyclodextrins may then be treated by porcine pancreatic α -amylase to produce modified oligosaccharide products.

The active site of PPA has been shown to consist of five D-glucose-binding subsites. The ability of PPA to productively bind modified residues at individual subsites will be reflected by the location of modified residues in oligosaccharide products. For example, it has been observed that subsite 3, the position of catalytic attack, is sensitive to sterically large substituents (8). As a result, PPA hydrolysis of these substrates does not form products which contain modifications in the reducing-end residue.

There have been several reports describing the action of PPA on modified substrates (2-11). Of these, only the report by Chan et al. (8) using hydroxyethylated amylose has included a substrate modified at C-2. The results from the hydroxyethyl derivative may be compared with results obtained from PPA hydrolysis of 2-deoxy- and 2-amino-2-deoxy-derivatives to assess the relative effects of sterically bulky, sterically small, and charged modifications on PPA catalysis.

PPA hydrolysis of mono-2-deoxy- β -cyclodextrin produces 2-deoxy-D-glucose, two isomers of 2-deoxymaltose, and three isomers of 2-deoxymaltotriose. These results may be interpreted to indicate that 2-deoxy-D-glucose undergoes productive binding (binding leading to hydrolysis) at all five subsites of the PPA active site. In contrast, hydrolysis of 2-aminoglycogen by PPA produces no modified monosaccharide, a single modified disaccharide, and larger products. These results are consistent with a proposed scheme of PPA attack shown in Fig. 8. Formation of 2²-amino-maltose indicates that 2-amino-2-deoxy-D-glucose may be productively bound at subsites 2 and 4, and formation of 2³-amino- and 2²-amino-maltotriose would indicate that productive binding of 2-amino- residues also occurs at subsites 5 and 1, respectively. Lack of formation of 2-amino-2-deoxy-D-glucose or of products modified in the reducing terminus suggests that the 2-amino- modification may not be productively bound at subsite 3. Results for C-2 modifications are summarized in Table I. Trends for these modified substrates agree with results for C-6 modifications (3-5,7,8,10,11), which indicate that bulky or charged groups are not accommodated at subsite 3 or at specific other subsites. Substrates containing sterically small substitutions may be productively bound at subsite 3, and at all other subsites for which binding has been examined.

PPA hydrolysis of substrates containing 2-deoxy-D-glucose has shown that modified oligosaccharide products are formed in an unequal distribution. We have measured changes in the composition of the modified maltotriose fraction during PPA hydrolysis of 2-deoxy- β -cyclodextrin and 2-deoxymaltotriose. Results indicate that modified residues affect rates

Figure 8. Proposed scheme for PPA hydrolysis of 2-aminoglycogen.

Open circles represent D-glucose. Filled circles represent 2-amino-2-deoxy-D-glucose. A slash denotes the reducing end.

PPA HYDROLYSIS OF 2-AMINOGLYCOGEN

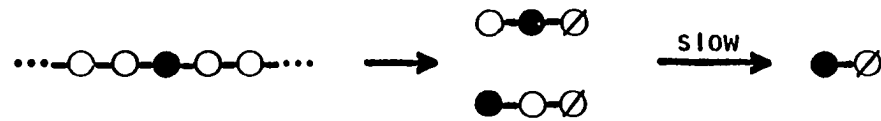


Table I. Productive Binding of Substrates Modified at C-2 to Subsites of Porcine Pancreatic α -Amylase

C-2 Modification	Porcine Pancreatic α -Amylase Subsite ^a					ref.
	1	2	3	4	5	
2-amino-2-deoxy-	+	+	-	+	*	this study
2-deoxy-	+	+	+ ^b	+	+	this study
2-O-hydroxyethyl-	+	-	-	+	*	8

^aSubsite numbering is from the reducing end as in ref. 8. Plus signs indicate productive binding is allowed. Minus signs indicate that binding is not allowed. Asterisks indicate that binding seems to occur, but has not been definitely demonstrated.

^bResults suggest that although productive binding at subsite 3 is allowed, it is not as favorable as binding of D-glucose.

of hydrolysis and bond-cleavage frequencies. This effect is particularly noticeable for productive binding of modified residues at subsite 3. The formation of modified disaccharides is the result of at least two hydrolytic steps, so that the distribution of the modification in disaccharide products depends on productive binding of the modified residue at more than one subsite. Since subsite 3 seems to show the greatest sensitivity to substrate modifications, the ratio of 2²-deoxymaltose to 2¹-deoxymaltose may provide a crude index of the relative productive binding of D-glucose and 2-deoxy-D-glucose at subsite 3. The hydrolysis of 2-deoxyglucosides by PPA contrasts sharply with acid-catalyzed hydrolysis. The rate of acid-catalyzed hydrolysis for 2-deoxy-D-glucosides is on the order of 2000 times greater than that of normal D-glucosides (23,24), whereas hydrolysis of 2-deoxy-D-glucosidic linkages by PPA is slower than for unmodified substrates.

The present study indicates that interactions involving the C-2 hydroxyl group, including hydrogen bonding, contribute to PPA catalysis but are not crucial. These experiments further suggest that the sensitivity of subsites 2 and 3 to bulky substituents is largely due to steric disruption. Interactions between enzyme and substrate are also disrupted by the sterically small, charged 2-amino- group. Catalysis by PPA seems to involve highly specific electronic and steric enzyme-substrate interactions at the point of hydrolytic attack, coupled with relatively less specific interaction throughout the rest of the extended active site.

These experiments have demonstrated the enzymatic production of modified cyclodextrins and oligosaccharides from corresponding substituted

polysaccharides. Such techniques may be useful in synthesis of biomimetic compounds and carbohydrase inhibitors. Further, these results, as part of a systematic study of amylose analogs, may also provide a picture of the steric, electronic, and hydrogen-bond interactions required for PPA hydrolysis.

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SECTION III.

THE EFFECT OF SUBSTRATE MODIFICATION ON
PORCINE PANCREATIC α -AMYLASE SUBSITE BINDING:
HYDROLYSIS OF AMYLOSE CONTAINING D-ALLOSE RESIDUES

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ABSTRACT

A modified amylose containing 10% tritiated D-allose residues has been hydrolyzed by porcine pancreatic α -amylase (PPA). This reaction produced a number of radioactive oligosaccharides of low molecular weight, including modified mono-, di-, and tri-saccharides, as well as larger products. Analysis of these products by chemical and enzymatic methods identified D-allose, two isomers of modified maltose, and isomers of maltotriose. These results may be interpreted in terms of current PPA models to indicate that D-allose residues may be productively bound at all five subsites of the enzyme active site. The distribution of modified residues in these products may further suggest that productive binding of D-allose at the subsite where catalytic attack occurs is less favorable than binding of D-glucose. These results are compared with results of a series of PPA substrates containing modifications at C-3 and at other positions. Trends observed in enzyme hydrolysis of these modified substrates reflect factors which contribute to PPA catalysis, with respect to steric, electronic, and hydrogen-bonding interactions between enzyme and substrate.

INTRODUCTION

Previous studies in this laboratory have determined the ability of porcine pancreatic α -amylase (PPA) to hydrolyze α -(1 \rightarrow 6)-branched substrates (1-2), hydroxyethylated amylose (3), oxidized-reduced amylose (4), and substrates containing sterically small substituents at C-2 (5) and C-6 (6). Other authors have also described the action of PPA on modified substrates (7-10), particularly in studies using PPA for structural analysis of polysaccharides. Previous experiments using substrates modified at C-3 have involved bulky and charged groups. In this report, we describe the hydrolysis of amylose containing 10% D-allose residues. Since this modification consists of inversion of configuration at C-3, these experiments may provide information about the effect of removal of the equatorial C-3 hydroxyl group, and may also identify important enzyme-substrate interactions which are disrupted by the introduction of an axial hydroxyl group at C-3. The distribution of modified residues within amylolysis products reflects the ability of these residues to bind productively at the five subsites of the PPA (11). By comparing results from various substrate modifications, the relative effects of charged substituents, sterically large groups, and small groups with altered hydrogen-bonding properties may be assessed. These results should provide insight into factors contributing to PPA catalysis.

EXPERIMENTAL

Materials

Amylose (Superlose) was obtained from Stein-Hall. Porcine pancreatic α -amylase (Boehringer-Mannheim, 10,000 U/mL) and *Rhizopus niveus* glucoamylase (Seikagaku Fine Chemicals, 35 U/mL) were commercial preparations, and were used without further purification. PPA contained no detectable α -glucosidase activity when assayed with *p*-nitrophenyl- α -glucoside. Tritiated sodium borohydride (40 mCi/mL, 252 mCi/mmol) was obtained from NEN-DuPont.

Methods

Paper chromatography was performed on Whatman 3MM paper using 1-propanol:water, 7:3 (solvent A) or nitromethane:acetic acid:ethanol:saturated aqueous boric acid 8:1:1:1 (solvent B). Separations were performed at 37°. Saccharides were hydrolyzed with 3M trifluoroacetic acid (TFA) for 2 h at 100° in sealed ampules. Sodium borohydride reduction of oligosaccharides for analysis was performed using the method of Sloneker (12). Tritiated samples on paper chromatograms were detected by autoradiography using En³Hance spray (NEN-DuPont), and radioactivity was determined by liquid scintillation spectrometry using a toluene cocktail.

Modified amylose containing D-allose residues was prepared from partially oxidized 6-O-trityl-2-O-benzoylamylose by reduction with sodium

borohydride, according to procedure of Kondo and Takeo (13). A sample of this product was hydrolyzed in 3M TFA, and the analysis of the hydrolysate by high-pressure liquid chromatography showed that 10% of the sugar residues had been converted to D-allose. Radioactive modified amylose was prepared by reduction of the partially oxidized amylose (150 mg) with [^3H]-sodium borohydride (125 mg, 2 mCi). Acid hydrolysis of the labeled product, followed by paper chromatography (20 h descent, solvent B) and autoradiography, showed two products with the paper chromatographic mobilities of D-allose and D-glucose, as well as a small amount of unhydrolyzed material. Results of liquid scintillation counting indicated that reduction of the oxidized amylose gave products with approximately 80% in the D-allo- configuration and 20% in the D-gluco- configuration.

PPA Hydrolysis of Amylose Containing D-Allose

Tritiated modified amylose (6 mg) was dissolved in 60 μL dimethyl sulfoxide:water, 9:1. This was diluted with 600 μL water, and buffer containing 200 mM sodium glycerophosphate, 100 mM sodium chloride, and 0.2% sodium azide, pH 6.9 (80 μL) was added. A sample (25 μL) was removed and spotted onto a paper chromatogram. An enzyme solution was prepared by adding 40 μL PPA to 80 μL buffer, pH 6.9, containing 20 mM sodium glycerophosphate, 10 mM calcium chloride, and 0.02% sodium azide. A portion of this enzyme solution (100 μL) was added to the substrate solution (715 μL) and the reaction mixture was incubated at 37°. Aliquots (25 μL) were removed at various times (1-96 h) and spotted onto the paper chromatogram.

At the end of the reaction time, 50 μ L was removed and reduced with sodium borohydride. The remainder of the reaction solution was streaked on to a second chromatogram. Ascending chromatography was performed (2 ascents, solvent A), and autoradiograms were prepared. The molecular size of D-allose-containing products was determined by comparison of their paper chromatographic mobilities with those of maltooligosaccharide standards. Mobilities of the modified and unmodified oligosaccharides were nearly identical using two ascents with solvent A.

RESULTS

Paper chromatography of products obtained from PPA hydrolysis from the modified amylose showed formation of radioactive monosaccharides, disaccharides, trisaccharides, and larger products. Labeled mono-, di-, and tri-saccharides were purified by paper chromatography (2 ascents, solvent A). The reaction contained substantial amounts of radioactive products that were relatively resistant to enzyme hydrolysis. A PPA digest of 6-deoxyamylose (d.s. = 0.15) performed under identical conditions, except that approximately half as much enzyme was used, contained less than one-third as much resistant material, which consisted of heptasaccharides and larger products (data not shown). We believe, but have not confirmed, that these resistant regions contain multiple D-allose residues, caused by nonrandom distribution of the modified residues.

Purified radioactive oligosaccharides were analyzed using descending paper chromatography (solvent B, 18-20 h). Chromatographic mobilities of A sample of the original PPA digest, which had been reduced with sodium borohydride, was subjected to total acid hydrolysis. Paper chromatography (solvent B) revealed that D-allose ($R_G = 1.4$) was the major radioactive product, with smaller amounts of D-glucose ($R_G = 1.0$), D-glucitol ($R_G = 2.1$), and D-allitol ($R_G = 1.9$). Rechromatography of the D-allose and D-allitol fractions confirmed the identities of these fractions. Isolation of D-allitol in this hydrolysate indicates that modified products containing D-allose in the reducing end are formed during PPA enzymolysis.

Paper chromatography (solvent B) of the monosaccharide fraction obtained from PPA hydrolysis of the modified amylose showed radioactive D-glucose with small amounts of radioactive D-allose. Chromatography of the labeled disaccharide fraction indicated that this fraction contained small amounts of maltose ($R_G=0.19$), plus a modified component with greater chromatographic mobility ($R_G=0.31$). Reduction of the disaccharide fraction produced maltitol ($R_G=0.35$) and a modified product ($R_G=0.57$). Total acid hydrolysis of the reduced modified disaccharide, followed by paper chromatography and autoradiography, showed D-allose as the major labeled product, with smaller amounts of D-allitol, D-glucose, and D-glucitol. This indicated that both possible modified maltoses [D-allosyl- α -(1+4)-D-glucose, and D-glucosyl- α -(1+4)-D-allose] are produced in the enzyme reaction. The ratio of (D-allose:D-allitol) released during acid hydrolysis was determined by liquid scintillation to be 9:1, indicating D-allose residues were located principally in the nonreducing end of the disaccharide. Thus, as seen for other experiments in which two isomers of modified disaccharide were produced (5,6), PPA hydrolysis favors formation of the disaccharide modified in the nonreducing residue.

The modified trisaccharide fraction obtained from PPA hydrolysis of modified amylose was examined by reduction, followed by treatment with *R. niveus* glucoamylase under conditions which gave complete hydrolysis of maltose, but no hydrolysis of maltitol. As a result, only the nonreducing residue is hydrolyzed from the trisaccharide, producing labeled monosaccharide and labeled reduced disaccharide products. The monosaccharide fraction obtained from glucoamylase hydrolysis of the reduced trisaccha-

ride contained labeled D-allose and a small amount of labeled D-glucose. This result indicated that the trisaccharide mixture contained maltotriose with D-allose in the nonreducing end. The reduced disaccharide obtained from glucoamylase hydrolysis was purified, and hydrolyzed with acid. D-Allose was the major product of this acid hydrolysis, with smaller amounts of D-glucose, D-glucitol, and D-allitol. These results indicate that the original trisaccharide fraction obtained from PPA hydrolysis of the modified amylose included maltotrioses containing D-allose primarily in the the middle and nonreducing residues, with a small amount in the reducing end.

DISCUSSION

The active site of porcine pancreatic α -amylase has been shown to consist of five subsites which bind a series of five glucose residues, with hydrolysis occurring between the second and third residues from the reducing end (11). When modified substrates are hydrolyzed by PPA, the structure of modified oligosaccharide products will reflect whether productive binding of the modified residues is allowed at some or all of the enzyme subsites. We have observed, for example, that subsite 3 (the subsite where hydrolysis occurs) is particularly sensitive to bulky modifying groups (1-3,7-9). As a result, modified oligosaccharides which are substituted at the reducing end are not formed.

The action of PPA on a modified amylose containing 10% D-allose residues leads to formation of D-allose and two modified disaccharides isomers. This indicates that productive binding of D-allose residues is allowed at subsites 1 through 4 of the enzyme active site. The disaccharide modified in the nonreducing-end residue is formed in approximately 9 times greater amount than the disaccharide modified in the reducing-end residue. This is consistent with previous results for amylose analogs containing 2-deoxy-D-glucose (5), 6-deoxy-D-glucose (6), and 6-deoxy-6-fluoro-D-glucose (6) residues. We have also obtained evidence for formation of a trisaccharide which contains D-allose in the nonreducing residue, which would require productive binding of D-allose residues at subsite 5. These results may be compared with results obtained for several unusual amylase substrates modified at C-3, as shown in Table 1. Residues

Table I. Productive Binding of Substrates Modified at C-3 to the Subsites of Porcine Pancreatic α -Amylase

C-3 Modification	Porcine Pancreatic α -Amylase Subsite ^a					ref.
	1	2	3	4	5	
D-allose	+	+	+ ^b	+	+	this study
3-O-hydroxyethyl-	+	+	-	+	*	3
3-O- α -D-glucosyl-	+	+	-	+	n.d.	1,2
3-O-phosphate-	-	-	-	+	-	10

^aSubsites numbered from reducing end as in ref. 11. Plus signs indicate that productive binding is allowed. Minus signs indicate that productive binding is not allowed. Asterisks indicate that productive binding probably occurs, but has not been definitely demonstrated. N.d. indicates that binding has not been determined.

^bProductive binding is allowed at subsite 3, but does not seem to be as favorable as binding of D-glucose.

containing bulky modifying groups (3-O-hydroxyethyl- or 3-O-D-glucosyl-) are not productively bound, in contrast to D-allose. Introduction of the sterically large, negatively charged phosphate group seriously affects enzyme-substrate interactions, and this modification is not productively bound except at subsite 4.

The results obtained from PPA substrates modified at C-3 are consistent with trends observed for modifications at C-2 and C-6. These trends are summarized as follows:

1. The effect of substrate modifications is greatest at subsite 3. Enzyme-substrate interactions at other subsites are relatively less specific.
2. Sterically large or charged modifications are not productively bound at subsite 3, and at other specific subsites.
3. Productive binding of sterically small modifications is allowed at subsite 3 and at other subsites. Productive binding of these modified substrates, however, seems to be less favorable than binding of D-glucose.

The sensitivity of subsite 3 to bulky and charged modifications is not surprising, since the site of catalytic attack might be expected to require a highly specific orientation. Results from bulky modifiers are very consistent (1-4,7-9), and indicate that subsite 3 is sensitive to substituents at C-2, C-3, and C-6. In addition, subsite 2 is sensitive to bulky modifications at C-2, and subsite 4 is sensitive to large modifications at C-6. This pattern of binding specificity may reflect the conformation of the substrate in the enzyme-substrate complex. Comparison of these results with results obtained from substrates modified with ster-

ically small groups suggests that the effect of bulky substituents originates from steric, rather than electronic disruption (5,6).

The active site of PPA has been shown by low-resolution x-ray diffraction to be a long crevice running down one side of the enzyme (14,15). The conformation of bound substrate has not been determined. The results of this study indicate that the action of PPA is not seriously affected by the presence of D-allose residues in the substrate. This may be consistent with binding of PPA substrates in a helical conformation. Inversion of configuration at C-3 of D-allose residues gives rise to an axial hydroxyl group which would protrude into the interior of a helix, a region that might be expected to be of limited accessibility to the enzyme. Binding of PPA substrates in a conformation having helical character may also be consistent with the observation that β -cyclodextrin is a competitive inhibitor of PPA (16), as well as being a poor substrate for the enzyme (17), and by the recent observation that maltoheptaose binds to the glycogen storage site of glycogen phosphorylase in a kinked left-handed helical conformation (18).

The PPA active site may resemble the hydrophobic cleft found for many enzymes, including lysozyme (19). The sensitivity of PPA to bulky or charged modifications at subsite 3 suggest that a highly organized ensemble of steric and electronic interactions are required at this subsite. Several authors, including Warshel and Levitt (19) and Wolfenden (20), have indicated that electronic interactions may be especially crucial for enzyme action in a hydrophobic enzyme environment. Interactions between PPA substrates and subsites other than subsite 3 are considerably less

sensitive to modification. Although binding requirements at subsites 1, 2, 4, and 5 are less stringent, their binding makes a major contribution to catalysis (21). Elucidation of the role of the multi-subsite PPA binding site remains central to understanding the catalytic efficiency of this enzyme.

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GENERAL DISCUSSION

Experimental results have been discussed in the three major sections of this dissertation. In this section, an attempt will be made to unify the conclusions of the individual sections and to present them, in an expanded and somewhat speculative form, in the light of current theories about carbohydrase action. In some instances, results obtained from other carbohydrate enzymes are included. These are presented for the sake of comparison, and are not intended as a comprehensive review of this diverse field.

The Role of Hydroxyl Groups in PPA Hydrolysis

Our results clearly suggest that individual substrate hydroxyl groups are not absolutely required for PPA catalysis. Results from the five modified PPA substrates examined in this study are summarized in Tables I and II. Four of these substrates contain modifications where hydroxyl groups were replaced with small substituents or where the stereochemistry involving a hydroxyl group was changed. For these modifications, productive binding was allowed at all subsites for which binding was determined. No evidence was obtained regarding the binding of 6-deoxy-D-glucose and 6-deoxy-6-fluoro-D-glucose residues at subsite 5. This subsite has generally been found to be insensitive to substrate modification, so that productive binding seems likely. The ratio of modified disaccharide isomers formed by PPA hydrolysis (Table II) may provide an approximate index of

Table I. Summary of Productive Binding of Modified D-Glucose Residues by Porcine Pancreatic α -Amylase

Modification	Porcine Pancreatic α -Amylase Subsite ^a				
	1	2	3	4	5
6-deoxy-	+	+	+ ^b	+	n.d.
6-deoxy-6-fluoro-	+	+	+ ^b	+	n.d.
D-allose	+	+	+ ^b	+	+
2-deoxy-	+	+	+ ^b	+	+
2-amino-2-deoxy-	+	+	-	+	*

^aSubsites numbered as in Fig. 1 of the General Introduction. Plus signs indicate that productive binding is allowed. Minus signs indicate that productive binding does not occur. Asterisks indicate that productive binding seems to occur, but has not been clearly demonstrated. N.d. indicates that binding has not been determined.

^bResults suggest that although productive binding at subsite 3 is allowed, it is not as favorable as binding of D-glucose.

Table II. Summary of Modified Product Formation Resulting from Porcine Pancreatic α -Amylase Hydrolysis of Modified Substrates

Modification	Product ^a			Ratio of Modified Disaccharides
	●-φ	○-φ	φ	
6-deoxy-	+	+	+	3-4
6-deoxy-6-fluoro-	+	+	+	11-15
D-allose	+	+	+	9
2-deoxy-	+	+	+	7-10
2-amino-2-deoxy-	+	-	-	-

^a(●-φ), nonreducing-end modified maltose; (○-φ), reducing-end modified maltose; φ, modified D-glucose. Plus signs indicate that the specified product is formed by PPA hydrolysis. Minus signs indicate the specified product is not formed.

how readily modified D-glucose residues are bound at subsite 3. Productive binding of 6-deoxy-D-glucose, 6-deoxy-6-fluoro-D-glucose, 2-deoxy-D-glucose, and D-allose is allowed, but does not seem to be favored. Surprisingly, the 6-fluoro- derivative is the most unfavorable substitution according to this interpretation. This observation has been discussed in detail in section I.

Similar studies from other carbohydrate enzymes vary considerably. In the course of experiments with PPA, it was observed that *R. niveus* glucoamylase, like PPA, will release D-allose, 6-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose, and 2-deoxy-D-glucose from modified substrates (data not shown). No 6-fluoro- derivatives were tested. Glucoamylase has also been shown to have a sensitivity to the bulky hydroxyethyl substituent similar to that of PPA (20). Uridine diphosphate deoxy-D-glucoses and deoxy-D-glucose-1-phosphates were examined by Biely et al. (108) and Zemek et al. (109-110) as substrates for yeast UDPG-glycogen glycosyltransferase and potato phosphorylase. In both instances, reaction rates were observed to decrease in the following order: D-glucose, 2-deoxy-D-glucose, 6-deoxy-D-glucose, 4-deoxy-D-glucose, 3-deoxy-D-glucose. In contrast, no incorporation of 2-deoxy-D-glucose was observed by Whelan using 2-deoxy-D-glucose-1-phosphate as a substrate for rabbit muscle phosphorylase (unpublished results cited in ref. 119). Experiments performed in this lab also indicated that 2-deoxyglycogen is not a substrate for the phosphorylytic reaction of rabbit muscle phosphorylase, and no incorporation of 3-deoxy-D-glucose into glycogen was observed using a crude mixture of 3-deoxy-D-glucose, and 3-deoxy-D-glucose- α - and - β -1-phosphates, using

either potato or rabbit muscle phosphorylase (data not shown). X-rays studies by Sprang et al. (120) on rabbit phosphorylase a in the T state have identified hydrogen bond interactions between substrate hydroxyl groups and the enzyme. These authors have also reported inhibition constants for several deoxy- and amino-sugars, as well as other analogs. Relative rates of enzymolysis of phenyl- β -deoxy-D-glucosides by *Aspergillus oryzae* β -glucosidase have been measured by Mega and Matsushima (121). The rates of hydrolysis decreased in the order D-glucose, 6-deoxy-D-glucose, 4-deoxy-D-glucose, 3-deoxy-D-glucose, 2-deoxy-D-glucose, with a difference in k_{cat} between D-glucose and 2-deoxy-D-glucose of six orders of magnitude. Roeser and Legler (122) observed rates of hydrolysis of 4-methylumbelliferyl-glycosides decreased in the order, D-glucose, 4-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose, 2-deoxy-D-glucose for *A. wentii* β -glucosidase, with k_{cat} again varying over six orders of magnitude.

Results obtained from PPA suggest that hydrogen bonding or similar interactions involving hydroxyl groups do play a role in catalysis, as evidenced by the uneven distribution of modified disaccharide products. The contribution from hydrogen bonding is not critical, and this contribution seems to be much less important for PPA catalysis than for hydrolysis by glucosidases. Actual rates of hydrolysis of modified substrates have not been determined for PPA in these experiments. If the effects of deoxy- and allo- modifications are assumed to be greatest at subsite 3, vast differences in the rates of enzymolysis of modified and unmodified substrates would give rise to highly skewed product distributions. We have examined product distributions for formation of 2-deoxy-disaccharides

and initial formation of 2-deoxy-trisaccharides (section II, Fig. 4). The observed distributions are slightly skewed, but do not seem to reflect differences in k_{cat} of 10^6 , as seen for β -glucosidases. The ability of PPA, glucoamylase, and potato phosphorylase to hydrolyze these modified substrates is probably due, at least in part, to the multi-subsite active sites of these enzymes. Binding of individual hydroxyl groups may play a relatively greater role for enzymes (such as glucosidases) that have smaller active sites.

The concept of catalysis driven by torsional strain has persisted for carbohydrase mechanisms since its proposal by Blake et al. (9). Early x-ray diffraction studies of lysozyme used the structure of an enzyme-trisaccharide complex to propose (by extrapolation) the structure of an enzyme-hexasaccharide complex in which enzyme subsites were fully occupied. These authors suggested that the enzyme-substrate complex contained sterically unfavorable interactions that could be resolved by forcing an N-acetylmuramic acid residue at the point of hydrolysis into a half-chair conformation. Recent binding, x-ray, and theoretical studies have suggested that the strained half-chair conformation is not necessary for binding (8,91-95). Calculations have also suggested that this strain requires substantial energy (10 kcal/mole), and that strain of this type may be readily relieved by small shifts in the atomic coordinates of the enzyme or the substrate (8). It was concluded from these calculations that torsional strain does not make a significant contribution to catalysis. Torsional strain, induced through hydrogen bonding, has also been proposed as a factor in PPA hydrolysis (4,5). The results from studies

using PPA substrates in which hydroxyl groups have been removed or their stereochemistry has been changed suggest that torsional strain induced through hydrogen bonding does not make a major contribution to catalysis. The contribution from hydrogen bonding of individual hydroxyl groups is not required for catalysis, and may be unrelated to torsional strain.

The Role of Electrostatic Interactions in PPA Hydrolysis

Only one charged PPA substrate (2-aminoglycogen) has been studied in this investigation, and charged substrates for PPA have not been widely studied in general. Present information (ref. 19 and section II of this thesis) indicate that charged groups do not bind productively at subsite 3 and at other specific subsites, and that binding depends on the size, charge, and location of the modification.

The active site of PPA has been shown by low-resolution x-ray diffraction to be a long crevice running down one side of the enzyme (59,60). Other carbohydrate enzymes, including lysozyme (9), hexokinase (123), and glycogen phosphorylase (120) have also been found to have cleft-like active sites which are relatively inaccessible to water. The dielectric constant of the interior of such active sites is generally considered to be small. Desolvation, in transferring reactants from bulk solvent to the protein interior, and highly organized interplay of electrostatic effects are believed to be important factors in enzyme-catalyzed reactions. Water-to-vapor distribution coefficients have provided one method for predicting the effects of desolvation of reactants in enzyme reactions.

Wolfenden (101) has recently published a brief review of desolvation studies. These studies predict that for enzyme reactions in which polar reactants are destabilized to less polar transition states, "catalysis by desolvation" may play an important role. In the mechanism proposed for PPA, however, the transition state is more polar than the starting material, so that reaction in a waterless enzyme might not be predicted to give rate accelerations. Two strategies are cited which might avoid this problem. 1) The mechanism proceeds in such a fashion that no intermediates more polar than the starting material are generated. Wolfenden cites formation of a covalent glycosyl-enzyme intermediate in the enzyme-catalyzed reaction, rather than a carbonium ion as in the acid-catalyzed mechanism. 2) The active site is designed to stabilize charges that are generated to a greater extent than solvent water. This may be illustrated by ionophores and metal-binding proteins which effectively sequester ions from water. Theoretical calculations (8,124,125) for reactions in aqueous solution and for enzyme-catalyzed reactions have suggested that electrostatic interactions are essential for the stabilization of charged intermediates in protein active sites which are inaccessible to water. In his model for the lysozyme active site (8), charged intermediates forming in a medium having a low dielectric constant are stabilized by dipoles induced on local polarizable groups, such as carbonyls. Young and Jencks (87) have estimated the lifetime of oxycarbonium ions formed during acid-catalyzed hydrolysis of model compounds to be 10^{-11} – 10^{-15} sec or less. Based on these results, he has proposed that the transition state for lysozyme has a structure intermediate between that of an oxycarbonium ion

and a covalent acylal intermediate, which would give it a short but significant lifetime.

Mechanisms involving covalent enzyme-substrate intermediates (4,5) and stabilized carbonium ion intermediates (2,3) have been proposed for PPA. In light of the foregoing discussion, the transition state for PPA might be considered to be either a highly stabilized carbonium ion, or as a destabilized covalent intermediate. These representations are difficult to distinguish from an experimental standpoint. Results of studies on PPA substrates containing charged modifications suggest that interactions around subsite 3 are highly sensitive to changes in the electronic environment. For the 3-phosphate modification (19), this disruption may be exerted over large distances. Formation or stabilization of the PPA transition state seems to require an well-organized ensemble of electronic interactions, as evidenced by its sensitivity to charged substrate modifications.

The Role of Steric Disruption in PPA Hydrolysis, and the Conformation of Bound Substrate

Productive binding of D-glucose residues containing sterically large substituents does not occur at subsite 3, for all experiments performed to date. This again suggests that highly ordered interactions are required at the point of hydrolytic attack. Bulky substituents attached to C-2 and C-6 will also prevent productive binding at subsites 2 and 4, respec-

tively. These subsites are adjacent to the hydrolytic subsite, and the observed steric effects may arise from this proximity.

The observed pattern of sensitivity to bulky modifications may reflect the conformation of the bound substrate; that is, groups which are sensitive to substitutions may be the groups that are most accessible to the enzyme in the enzyme-substrate complex. The conformation of the bound substrate in the PPA active site crevice has not been determined. Several lines of evidence suggest that the bound substrate may have some helical character. β -Cyclodextrin is a competitive inhibitor for PPA (23), as well as being a relatively poor substrate (22). The PPA active site cleft has appropriate dimensions to bind β -cyclodextrin with the plane of the torus parallel to the longitudinal axis of the crevice, and the external surface of the torus directed into the cleft. Binding of a helical substrate is also supported by the preference of amylose polymorphs for helical conformations (126,127). A helical substrate is also consistent with our observation that D-allose does not seriously affect PPA action. The axial C-3 hydroxyl of D-allose would protrude into the interior of a helix, and thus might be expected to be of limited accessibility to PPA and to have little effect on productive binding. Left-handed and right-handed helices were examined, therefore, to determine if binding of amylose in these conformations might account for the observed steric sensitivity. Line drawings of these helices, generously provided by Dr. Alfred French, are shown in Fig. 3 and Fig. 4. These computer models (127) represent 6-fold helices with an 8 Å repeat distance, based on the structure of V amylose. Two slight differences appear in these models. First,

Figure 1. Six-fold right-handed helix of amylose, with repeat distance of 8 Å. A) Axial view. B) Side view.

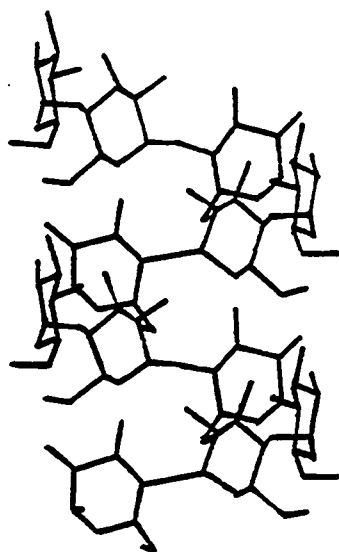
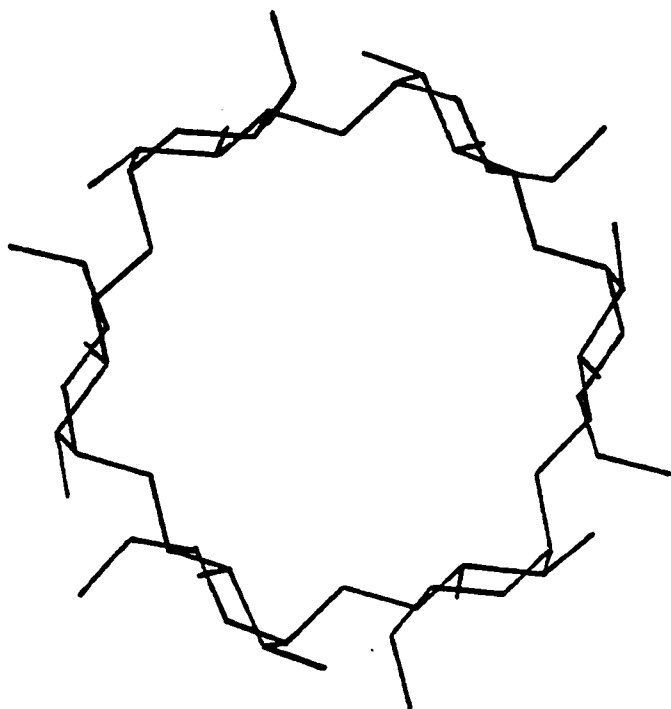
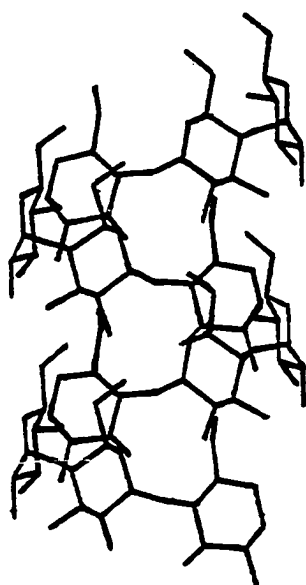
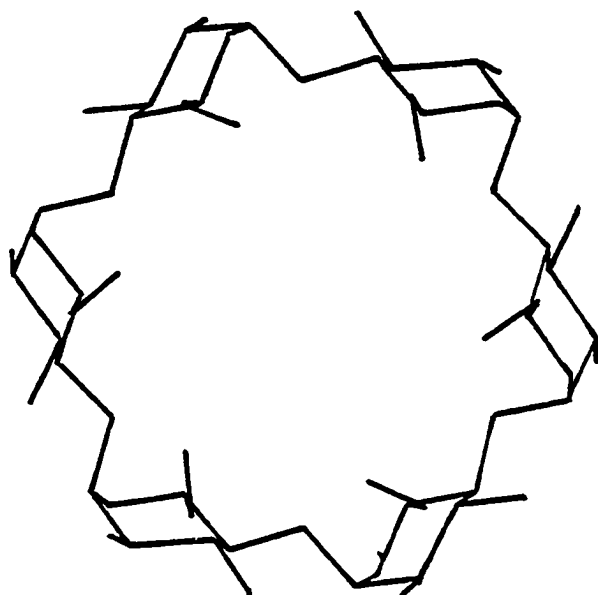


Figure 2. Six-fold left-handed helix of amylose, with repeat distance of 8 Å. A) Axial view. B) Side view.



the C-6 hydroxyl is directed out of the helix for the right-handed helix, but not in the left-handed helix. This results from short contact distances at 0-6 of the right-handed helix, which may be relaxed by increasing the repeat distance. Second, the glucose residues are tilted differently in the two helices, with the secondary hydroxyl groups being more accessible in the left-handed helix, and the C-6 hydroxyl being more accessible in the right-handed helix. These considerations suggest that a regular left-handed helix will favor accessibility of secondary hydroxyls, and that a regular right-handed helix will favor accessibility of primary hydroxyls, but that neither helix will give increased accessibility to both types of groups. Manipulation of molecular models suggests that introduction of a kink into either type of helix can produce a conformation with improved accessibility of the C-6 hydroxyl at subsite 4 and the C-2 hydroxyl at subsite 2. For a left-handed helix, this kink can be introduced at subsite 4; for a right-handed helix it can be introduced at subsite 2. This kink tends to decrease the repeat distance for that turn of a right-handed helix, however, and tends to introduce unfavorable contact interactions. Introducing the appropriate kink in a left-handed helix tends to increase the helix repeat distance for that turn. This latter helix may also have an advantage in that it makes the interior of the helix more accessible to enzyme functional groups, such as the basic group which has been proposed to protonate the oxygen of the glycosidic linkage during hydrolysis (see Fig. 2 in the General Introduction). Based on these considerations, a left-handed helix may be more favorable. A

left-handed helical conformation has been reported for maltoheptaose bound to the storage site of glycogen phosphorylase (128).

The Role of the Multi-Subsite PPA Active Site

It has been observed in these studies that interactions at subsite 3 of the PPA active site are very sensitive to steric and electrostatic disruption, and, to a lesser extent, to hydroxyl group removal. The requirement for specificity of interactions at the remaining sites, on the other hand, seems to be much less stringent. Binding at these subsites, however, clearly contributes greatly to the overall catalytic rate as shown in the data of Prodanov et al. (80), which show that the turnover number of PPA increases by a factor of 8500 as substrate size increases from three to five D-glucose residues.

The role of enzymes as "entropy sinks" has been widely considered (129,130), and one possible role for the extended PPA active site could be in aligning the substrate with enzyme catalytic groups. Thoma (2) has estimated an upper limit of the pseudo-first-order rate coefficient for acid hydrolysis of maltose to be 10^{-8} sec^{-1} . The enzyme catalytic rate coefficient is approximately 1.2×10^3 , leaving an acceleration factor of 10^{11} or greater which must be accounted for in order to explain enzyme catalysis. Based on entropic considerations for aligning an amylase substrate with two catalytic groups, Thoma estimates that the rate constant may be increased by up to a factor of 3×10^7 . Thus, entropic advantages may account for a substantial portion of PPA rate acceleration.

It is possible that rotational and translational motion of a D-glucose residue at subsite 3 are increasingly restricted for binding of progressively larger substrates (up to maltopentaose), however, correlation between substrate size and entropy loss has not been reported for PPA.

A second factor which may be related to the role of the extended active site is based on the concept of complementarity between enzymes and transition states. According to this theory, maximum binding interactions between enzyme and substrate occur when the substrate is in the transition state, rather than the ground state (130-132). In a model proposed for lysozyme (10), the polysaccharide is initially bound, then as the N-acetylmuramic acid residue at subsite D begins to approach a half-chair transition state, the conformation of the polysaccharide changes. This conformation change allows greater contact between enzyme and substrate at subsites D, E, and F, and increases the binding energy. Chipman and Schindler have estimated that this mechanism may produce a rate acceleration of 2500-fold for the lysozyme reaction. This mechanism is similar in some ways to the torsional strain models. Both are based on the idea that enzymes show a greater affinity for the reaction transition state than for the starting material. The "binding-site complementarity" model suggests that increased binding energy rather than distortion provides the driving force, and that, in the case of lysozyme, these interactions are spread over a large region of the substrate rather than being highly localized. No experiments have been performed to study the applicability of this theory to catalysis by PPA.

Conclusion

The results of experiments described herein may be compared with the action of PPA on other modified substrates. The specificity of PPA hydrolysis has been interpreted in the light of current theories of carbohydrase action. Conclusions from this study may be summarized as follows:

1. The effect of substrate modification is always greatest at subsite 3 of the PPA active site. Enzyme-substrate interactions at other subsites are relatively less specific.
2. Sterically large substituents are not productively bound at subsite 3, or at specific positions at other subsites. A comparison with results obtained for small substituents suggests that this effect arises principally from steric disruption of enzyme-substrate interactions.
3. Charged substituents are also not productively bound at subsite 3 and at specific positions at other subsites. This effect varies depending on the size, charge, and location of the modifying group. The effect of charged modifications may be exerted over large distances.
4. Small substituents in which hydrogen-bonding capabilities have been altered are productively bound at all subsites which have been experimentally observed. Productive binding of the modified residues at subsite 3 is less favorable than binding of D-glucose.
5. Electronic effects, including acid-base catalysis and/or electrostatic stabilization, seem to make substantial contributions to PPA catalysis. Other factors, such as transition state complementarity, may make

an undetermined contribution. Torsional strain does not seem to be a major contributing factor in PPA hydrolysis.

6. The observed enzyme specificity may be related to the conformation of substrate during binding. This specificity is consistent with binding of an irregular helical conformation.

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